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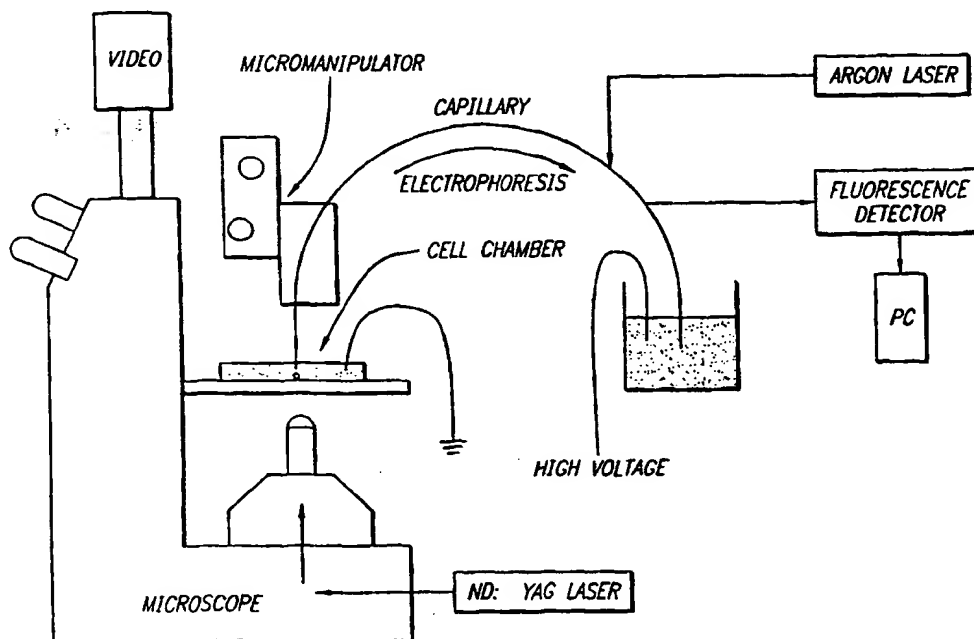
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(54) Title: FAST CONTROLLABLE LASER LYSIS OF CELLS FOR ANALYSIS

(57) Abstract

Fast lysis of a single cell (46) or cellular component thereof is performed by generating a shock wave in a medium in which the cell (46) or cellular component thereof is positioned. The cell (46) or cellular component thereof is either positioned by laser tweezers or cultured as an adhered cell or cellular component thereof to minimize manipulation trauma. The disclosed method completely lyses a single cell (46) or cellular component thereof in a controllable manner in milliseconds or less followed immediately by the loading of the cellular contents into a capillary (22) for analyte separation and detection. The cell (46) or cellular component thereof is adjacent the inlet of an electrophoretic column

(22) through which a gravity siphon flow of the medium is maintained. The lysed contents of the cell (46) or cellular component thereof enter the electrophoretic column (22) in less than 33 msec, and are separated and analyzed by a fluorescence detector (42). The method takes advantage of the shock wave produced by a highly focused laser pulse from a Nd:YAG laser (18) which is created in a medium adjacent to the cell (46) or cellular component thereof. In the illustrated embodiment, the laser pulse is focused in the slide (28) at or near a glass-to-buffer interface of a cell chamber in which the cell (46) or cellular component thereof to be lysed has been cultured.



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Fast Controllable Laser Lysis of Cells for Analysis

Background of Invention

1. Field of the Invention

The invention relates to the field of analytical chemistry in the areas of cellular biochemical and biomedical analysis and in particular to a laser microsurgery apparatus and method performed in less than one second for controllably lysing a single cell or selected cells and then collecting all or a selected portion of the cellular contents for immediate chemical analysis.

2. Description of the Prior Art

Dramatic progress in our understanding of biological processes has been made possible by studies of single cells. Research directed at the individual cells of an organism has utilized recent technological advances in optical and chemical methods. Laser-based techniques for manipulation of single cells and subcellular structures have enabled the performance of surgery on single cells. Ultrasensitive chemical analysis methods have now been used in biochemical studies of single cells. New knowledge gained from such single-cell studies is already finding medical and commercial applications. It can be expected that technology for the manipulation and analysis of single cells will play an important role in such areas as biomedical research, drug discovery, diagnosis of disease, and medical treatment.

In the past decade the tools of the analytic chemist have been applied to biochemical studies of living cells. For example, single neuronal cells from nonmammalian species have been analyzed through the use of capillary electrophoresis. By virtue of the large size of these neuronal ganglion cells subcellular measurements have been possible. Typically these cells are approximately 0.1 to 0.2 millimeters in diameter. In the prior art technique, the end lead of a capillary is etched to a fine point and used to sample

cytoplasm from a cell or disrupted cellular fragment. Cytoplasmic contents are separated within the capillary and either detected on or off line.

For smaller mammalian cells in the range of 10 to 15 microns in diameter, the entire cell is loaded into the capillary and then the cell is lysed with a

5 hypotonic or detergent-containing buffer. The cellular contents are separated in the capillary and detected by a variety of methods such as laser induced fluorescence or eletrochemical detection. However, cellular lysis is not easily controllable either as to the time at which it takes place or over the duration during which it occurs. For measurements on mammalian cells, the temporal
10 resolution of the measurement techniques and the effects of perturbing the cell prior to complete lysis are important issues to consider. Lysis is defined for the purposes of this specification as the disruption of at least a portion of the plasma membrane with the release of at least a portion of the cellular contents. The manner in which the cell is lysed will govern the time which is
15 required to terminate the biochemical reactions in progress, that is the manner of lysis will govern the temporal resolution of the biological measurement. Also, the manner of lysis will influence cellular processes occurring during the period of sampling. Many biological events take place on time scales of seconds or less. The enzymes typically have turnover
20 numbers on the order of one to ten thousand per second. Metabolite concentrations can change greater than ten-fold in one second. Accurate measurement of such cellular properties by analytical techniques requires then that complete cell lysis occurs rapidly in comparison to the rate of change of the measured parameter. If disruption of the cell membrane occurs
25 at a rate which is too slow, significant changes in the parameter can occur during the very lysis of the cell resulting in an inaccurate view of the actual physiological state of the cell.

In addition, membrane permeabilization during lysis results in the influx of extracellular ions such as calcium (Ca^{2+}), activating many enzymes including
30 kinases, phosphatases, proteases and nucleases. Even after disruption of the plasma membrane, biochemical processes will proceed until reactions are terminated, that is by separation of the reactants or denaturation of the

molecules. Therefore, in order to accurately measure many cellular processes, complete cell lysis must be performed within milliseconds or less. The prior art chemical lysis is incapable of providing this speed in any manner which is controllable so that effective cellular analysis is possible.

5 Another important consideration in making whole cell measurements is the effect caused by manipulation of the cell prior to sampling. In studies of nonadherent or free floating cells using capillary electrophoresis the use of electroosmotic flow to move the cell into the capillary inlet may impact the cell and hence the process which is to be measured. A large body of literature
10 exists on the biological effects of electrical fields. Unfortunately, most of the literature addresses AC rather than static fields so that the effects of low DC electric field strengths on cellular physiology have not been well characterized. However, there is no doubt that there is some effect. Application of an electric field with a gradient of the order of 1 - 2 kV/cm, can
15 induce permeabilization of the cell membranes, a phenomenon known as electroporation. The potential gradients below 1 - 2 kV/cm, which are generally used for capillary electrophoresis, namely on the order of 400 V/cm, have important effects on cellular physiology. At an electric field strength of 167 V/cm for a five millisecond duration, there were localized increases in
20 cellular permeability with concomitant influx of calcium ions (Ca^{2+}) has been demonstrated. Such calcium ion influx can activate numerous cellular processes that may affect biochemical measurements.

In order to move a cell into a capillary, investigators have induced electroosmotic flow using potential gradients varying from 10 V/cm to 300
25 V/cm. While potential gradients in the range of 10 - 20 V/cm are unlikely to perturb cellular physiology, higher field strengths most certainly will. Adherent cells or cells that adhere to a substrate, such as a glass slide or pipette are not amenable to manipulation by electroosmotic flow or hydrodynamic flow without first removing them from their attaching substrate.
30 However, the mechanical stress induced by such removal can trigger a variety of cellular responses. Nearly all cells express abundant adhesion proteins at their surfaces. Structures known as focal adhesions provide a

structural link between the cytoskeleton and the extracellular matrix. Focal adhesions consist of integrins and other proteins which are linked to a variety of intracellular signal transduction pathways. Mechanical stresses act through these membrane components to activate numerous enzymes including tyrosine kinases, serine/threonine kinases, G-proteins, proteases and others. Activations of these pathways trigger immediate and long term changes in the cellular physiology. For this reason mechanical manipulation, especially with adherent cells, prior to the time of sampling may interfere with measurement of the cellular biochemistry.

- 5
- 10 What is needed is a method to completely or at least partially lyse a single cell in milliseconds or less in a manner which is controlled both as to time and place and which does not affect the cellular physiology prior to lysis followed by loading of the cellular contents into a device for analysis such as a capillary for analyte separation and detection.

15 Brief Summary of Invention

- The invention is a method for lysing and analysis of the contents of a selected cell or cellular component thereof comprising the steps of controllably selecting at least one of a plurality of cells or cellular component thereof in a medium. The selected cell or cellular component is disrupted or lysed with a laser generated shock wave in the medium or in a substrate or glass surface some distance from the cell. At least a portion of the contents of the cell or cellular component is collected. The collected contents are then analyzed. In the illustrated embodiment the contents of the cell or cellular component thereof is collected within one second or less of lysis of the cell. This is of utility when the cell is living so that the biological reactants can be analyzed in the state which they had obtained at the instant of lysis. In fact the contents are collected within 33 msec or less of lysis of the cell and it is believed that collection is delayed only by the time for the cell or cellular component to porate or open, which is believed to be a few microseconds or in the range of 1 - 10 microseconds of lysis of the cell. As a result, the time between disruption of cellular biochemical activity and the cessation of reactions of the
- 20
- 25
- 30

cellular contents is minimized, yielding a very accurate view of the cellular contents immediately prior to lysis. However, the invention is applicable to other applications as well, such as the lysis or rupture of dead cells, where the time between lysis and cessation of reactions is not as critical.

- 5 The step of controllably selecting at least one of a plurality of cells or cellular components comprises the steps of identifying and determining the position of the selected cell or cellular component thereof. Either the identified cell or component can be brought to the position near the focal point of the laser beam and collection inlet, or the position of focal point of the laser beam and
10 collection inlet can be brought near the identified cell or component.

One method to controllably position the selected cell or cellular component in the medium comprises the step of adhering the cell or cellular component thereof to a substrate disposed at least adjacent to the medium.

- Where the cell or cellular component is free floating the step of controllably
15 positioning the selected cell or cellular component in the medium in another embodiment comprises the step of temporarily holding the cell or cellular component in a position in the medium by a laser microbeam optical tweezers, or temporarily holding the cell or cellular component in a position in the medium by adhesion of a mechanical micromanipulator to the cell or
20 cellular component. The cell or cellular component can be attached to a pipette or microprobe either by suction or by means of electrical or chemical adhesion.

- The process may be practiced under human control or by automated software control. For example, the field of view within a target zone adjacent to the
25 inlet of the analysis device is optically monitored by a video system coupled to a computer provided with pattern recognition software or by a human operator. When a target cell is properly positioned in the target zone and identified as a desired target, a laser pulse is generated that produces a shock wave which lyses the cell followed by immediate collection into the
30 analysis device.

Still further the free floating cell or cellular component can be controllably positioned in the medium by positioning the cell or cellular component in a

confined enclosure such as the inlet to the analysis device or a holding enclosure in which the cell is positioned.

The step of collecting at least a portion of the contents of the cell or cellular component thereof may further comprise the step of stopping the reactions of
5 biochemical reactants disrupted from the selected cell or cellular component thereof to permit subsequent analysis of the biochemical reactants in the state which existed approximately at the time of disruption.

In the illustrated embodiment the step of collecting the contents of the disrupted cell or cellular component in the analysis device comprises the step
10 of collecting the cell or cellular component in an electrophoretic column. The collected contents are electrophoretically separated and analyzed using laser induced fluorescence.

The step of disrupting the selected cell or cellular component with a laser generated shock wave in the medium comprises in one embodiment the step
15 of focusing a pulsed laser beam at a position proximate to the cell or cellular component, but without focusing on the cell or cellular component and then generating the shock wave.

In another embodiment the pulsed laser beam is focused directly in or on the cell or cellular component. The pulsed laser beam defines an opening in the
20 cell or cellular component to obtain only cytoplasmic contents therefrom.

In the illustrated embodiment the step of collecting at least a portion of the contents of the disrupted cell or cellular component thereof in the analysis device is by means of fluid flow of the medium, and in particular by means of siphon fluid flow of the medium. Collection can also be effected by means of
25 electrophoresis through the medium, by means of force from the shock wave impacted on the contents, and by means of electroosmotic force.

The invention is alternatively defined as an apparatus for lysing and analysis of the contents of one of a plurality of cells or cellular components thereof comprising a cell selector to controllably select at least one of the cells or
30 cellular component thereof. A laser generates a pulse to lyse or rupture the selected cell or cellular component. An analysis device is provided to analyze

the contents. A collector captures or delivers at least a portion of the contents of the lysed cell or cellular component to the analysis device.

The collection after lysis and the lysis itself is fast. The collector delivers at least a portion of the contents of the lysed cell or cellular component to the

5 analysis device within one second of lysis of the cell or cellular component.

Where applied to a living cell the contents of the lysed cell or cellular component is delivered to the analysis device within 33 msec of lysis of the cell or cellular component and in all probability within 1 - 10 microseconds of lysis of the cell or cellular component.

10 The analysis device may be any microanalytical chemical, electrical or electrochemical device or technology such as a means for performing polymerase chain reactions on the contents, a means of separating analyte molecules such as a gel or capillary electrophoretic column, and a means for detecting separated analytes such as a gel reader or a laser induced

15 fluorescence detector.

The invention is also defined as a method for fast lysing and analysis of the contents of one of a plurality of cells or cellular components thereof comprising the steps of controllably selecting at least one of the plurality of cells or cellular components thereof by relative placement of the selected cell

20 or component adjacent to an inlet orifice of a pipette. The selected cell or cellular component thereof is lysed with a laser generated pulse. At least a portion of the contents of the lysed cell or cellular component thereof is collected in the pipette within one second of lysis for subsequent analysis.

Any further substantial biologic reactions in the contents are stopped after

25 lysis.

The invention and its various embodiments, now having been summarized, may be better understood by viewing the following drawings wherein like elements are referenced by like numerals.

Brief Description of the Drawing

30 Fig. 1A is a schematic diagram of a system used for fast controllable cell lysis according to the invention.

Fig. 1B is a magnified diagrammatic side view of the area in the vicinity of the inlet of the capillary used in Fig. 1A showing the position of the capillary inlet with respect to a single cell and with respect to a location of a focused laser pulse.

- 5 Fig. 1C is a magnified diagrammatic bottom view of the area in the vicinity of the inlet of the capillary used in Fig. 1A and B showing the position of the capillary inlet with respect to a single cell and with respect to a location of a focused laser pulse.

- 10 Figs. 2A - D are video still images taken before and after lysis. Fig. 2A is a microphotograph of a cell taken immediately prior to lysis of the cell.

Fig. 2B is a video frame at the instant of the start of lysis when applied according to the invention. The focal point of the laser is just below the surface of the cover slip so the cell is slightly out of focus. The bright image shown by the arrow is the reflected image and not the actual laser pulse.

- 15 Fig. 2C is a video frame of the cell 33 msec after lysis. Cell remnants and secretory granules can be seen. The divot in the glass caused by the interaction of the laser with the glass substrate is also visible.

- 20 Fig. 2D is the same video frame as shown in Fig. 2C but with the focal plane adjusted to match that as shown in Fig. 2A in order to better resolve the cell remnants in the image.

Figs. 3A - C are a series of video still frame images taken every 33 msec of a single cell positioned at the inlet of a capillary. Fig. 3A shows the cell immediately prior to the laser pulse being applied.

- 25 Fig. 3B shows a circular wave emanating from the point of a laser pulse which has not yet interacted with the cell.

Fig. 3C shows the cell subsequent to lysis. The cell remnants and contents have been loaded into the capillary and are no longer visible in the image. The laser induced defect in the cover slip is visible.

- 30 Fig. 4 shows four stacked electropherograms of single cells and a free acid standard containing the fluorophores, fluorescein, and/or Oregon Green.

Fig. 5 is an electropherogram of two cells loaded with both fluorescein and Oregon Green, which cells were lysed in sequence and then loaded in

sequence into the capillary. The subscript "1" indicates the peaks from the cell lysed first and the subscript "2" indicates the peaks from the cell lysed second in time.

The invention and its various embodiments may now be understood by
5 turning to the following Detailed Description of the Preferred Embodiments.

Detailed Description of the Preferred Embodiments

The illustrated method completely lyses a single cell in a controllable manner in milliseconds or less followed immediately by the loading of the cellular contents into a capillary for analyte separation and detection. The method
10 takes advantage of the shock wave produced by a highly focused laser pulse which is created in a medium adjacent to the cell. The laser could also be focused (a) in the glass slide adjacent to the cell, (b) in a substrate disposed on the glass slide, (c) in the fluid at the interface of the glass slide or substrate, or (d) in the fluid adjacent to the target cell in the case where the
15 cell is free floating or being held by optical tweezers. In the illustrated embodiment the laser pulse is focused in the glass substrate at or near a glass-to-buffer interface of a cell chamber in which the cell to be lysed has been cultured or attached. It must be understood that the laser pulse may be focused at other locations such as on the cell wall or membrane itself, where
20 for example a cytoplasmic puncture is desired to remove just a portion of the cell contents, or may be focused at any position in the fluid surrounding the cell in the cell chamber. In the illustrated embodiment a 5 nsec laser pulse is used to cause a localized plasma. The formation of the plasma at the focal point is believed to produce a cavitation bubble in the fluid medium nearby.
25 The expansion and collapse of this cavitation bubble generates a supersonic or near supersonic shock wave traveling up to 2,000 m/sec. For a cell 20 microns in diameter such a shock completely traverses the cell in 10 nsec. Therefore for a cell 20 microns away from the focus, all the energy needed for lysis is delivered or impinges on the cell within about 25 nsec of initiation of
30 the shockwave by the laser pulse. The cell explodes or is lysed in response in what is believed to be a few microseconds, i.e. in the range of 1 - 10

microseconds. The interaction of the shock wave with the cell rapidly disrupts the cell membrane thereby quickly releasing the cellular contents. Although the physics of the formation of the plasma and its cavitation bubble is not clearly understood, it is believed that there is a electron avalanche unique to generation of the localized plasma which produces the cavitation bubble. The illustrated embodiment also includes other mechanical stresses and optically induced damage that are generated by focused laser beams, which may not be associated with plasmas.

Fig. 1A is a block diagram of the illustrated embodiment of a system in which fast controlled cell lysis is performed in combination with analysis by capillary electrophoresis. It must be understood that many components now known or later devised may be substituted into the system of Fig. 1A to perform equivalent functions. For example, although the illustrated embodiment contemplates laser induced fluorescence detection of analytes in an electrophoretic column, any measurement device or detection mechanism capable of analyte detection or analysis may be used.

The system, generally denoted by reference numeral 10, is comprised of a microscope 12 having a video ocular readout 14 displayed on a CRT screen 16 or recorded in a videotape recorder or digital recorder (not shown). In particular, as will be described below digital storage of the images and pattern processing in a computer system for automated cell processing and analysis is specifically contemplated as being within the scope of the invention. The CCD video camera system 14, 16 recorded the real time bright field image of cell 46 every 33 milliseconds. Lysis was determined from the appearance of the cell after the laser pulse. In most cases, the cell membrane was totally disrupted, leaving behind only a remnant of the membrane attached to cover slip 36 with the cellular contents suspended in the immediate vicinity of the cell as depicted in Fig. 2C. In the illustrated embodiment, electrophoresis was operator-initiated upon visualization of cell lysis, which was estimated to be a 300 msec response time. It is to be expressly understood that computer-controlled pattern recognition could be substituted for even faster

reaction times or electrophoresis could be triggered directly off the laser pulse.

A pulsed Nd:YAG laser 18 is directed to microscope objective optics 20 of microscope 12. The laser system used for the cell lysing experiment included
5 a frequency doubled Q-switched Nd:YAG laser such as manufactured as the Surelite I, by Continuum of Santa Clara, California. The laser was used to generate a single laser pulse of 10 to 100 (J with a 5 nsec pulse with a 532 nm wave length. The laser beam from laser 18 was directed into microscope 12, namely Axiovert 135 manufactured by Zeiss of Thornwood, New York.

10 The laser pulse was focused to approximately 0.3 to 0.4 microns at its waist 50 as shown in Fig. 1B using microscope objective 20 (63 x, 1.25 n.a. Zeiss) at the interface of the cell chamber cover slip 36 and buffer solution 54. Cell 46 which was to be lysed was positioned 20/30 microns laterally to focal point 50 of the laser pulse. It is to be expressly understood, however, that in
15 another embodiment direct interaction would be arranged, such as where the laser parameters were adjusted only to open a hole in the cell membrane to expel the cytoplasmic contents, leaving the nuclear material within. It must be understood that other types of lasers other than the one illustrated here may also be used to generate the shock wave. In addition to lysis of the cell, the
20 nucleus of the cell which has been isolated or removed from the cell may by manipulated to recover the DNA or other nuclear material.

A fused silica capillary column 22 is positioned by means of a micromanipulator 24 which positions proximate inlet 26 of capillary 22 above a cover slip or slide 28 positioned on microscope stage 30. The buffer
25 solution around the cell and above the cover slip is electrically grounded. The distal end 32 of capillary 22 is disposed in an electrolyte or buffer bath 34 in order to create a gravity siphon pressure through capillary 22 at proximate end 26. A high voltage power supply, model CZE 1000R manufactured by Spellman of Plainview, New York, was used to drive the electrophoresis in
30 column or capillary 22. Fused silica capillary 22 had a 50 micron inner diameter and 360 micron outer diameter. The lumen walls were coated with a proprietary neutral coating manufactured by Supelco of Phoenix, Arizona.

The coating was used to minimize the electroosmotic flow and thus shorten the migration times for the negatively charged, fluorophores used in these experiments. The total length of the capillary was 90 to 100 cm. The detection window was about 75 cm from inlet 48. Electrophoresis was performed in a biologically compatible buffer. The cell chamber served as an inlet reservoir and was held at ground potential. The outlet reservoir was held at 15 to 18 kV. Distal outlet 32 of capillary 22 was placed 5 centimeters below inlet 48. Inlet 48 of capillary 22 was used as a micropipette for introducing the cellular contents into capillary 22 after cell lysis.

After removing 5 mm of polyimide coating from capillary 22 above inlet 48, inlet 48 was mounted perpendicularly to cover slip 36 on micromanipulator 24. Micromanipulator 24 enabled precise positioning of capillary lumen 52 with respect to cell 46 to be lysed and loaded into capillary 22. Capillary 22 was washed with fresh buffer A for one to two minutes after every run.

Gravitational siphon fluid flow was used to load capillary 22 with a fluorescein/Oregon Green free acid standard. The loaded volumes were calculated from Poiseuille's equation.

Capillary 22 includes an inspection window 38 upon which an excitation beam from Argon laser 40 is exposed. Optical window 38 in the polyimide coating of capillary 22 was created 75 to 85 cm from inlet 48. Capillary lumen 52 was interrogated by a focused laser beam from Argon ion laser 40. Fluorescence data were collected at a right angle to capillary 22 and to the laser beam from laser 40 with a microscope objective of 40X with a 0.75 n.a. Plan Fluor manufactured by Nikon of Melville, New York. The light was measured with a photomultiplier tube, a PMT R928 made by Hamamatsu, of Bridgewater, New Jersey after spectral filtering with a 488 notch filter manufactured by Kaiser Optical Systems of Ann Arbor, Michigan and filtering with a band pass filter 535 DF55 made by Omega Optical of Brattleboro, Vermont. The photomultiplier current was amplified and converted to a voltage with a preamplifier then digitized by a data acquisition board in a personal computer 44. The data were plotted and peak areas calculated using Origin written by Microcal of North Hampton, Massachusetts. Induced fluorescence is

detected by fluorescence detector 42 to create the electropherograms of Figs. 4A-D through the use of a personal computer 44. Bath 34 is electrically coupled to high voltage source 36 to provide the electrophoretic force to cause analyte separation in capillary 22.

- 5 Fig. 1B is a highly enlarged side view of proximate end 26 of capillary 22 showing a single cell 46 disposed on cover slip 36 and positioned adjacent inlet 48 of proximate end 26 of capillary 22. Laser 18 is focused in a focal area, diagrammatically depicted as focal area 50 either in cover slip 36 or just below the fluid-to-cover slip interface on its upper surface. Fig. 1C is a
10 bottom view of the enlarged depiction of Fig. 1B showing the planar relationship of the same elements and in particular the spatial overlap of cell 46 to inlet 48 and the proximity of laser focus 50.

- By positioning inlet 48 of capillary 22 directly above cell 46 prior to lysis, as shown in Fig. 1C the cellular contents of the lysed cell are loaded into lumen
15 52 of capillary 22 by combination of the gravity siphon and electrophoresis at lysis. The force of the shock wave also conveys momentum to the cell fragments which drive them into the lumen of the capillary. As will be shown below the time required between the instant of the beginning of lysis and the loading within lumen 52 is significantly less than 33 msec. The absence of
20 any manipulation of cell 46 prior to lysis and the extreme rapidity of the lysis itself results in minimal physiological effects on cell 46 up to the time of sampling. Once cell 46 is lysed, cellular reactions are terminated by diffusion, turbulent mixing or electrophoretic separation of reactants thus making more accurate snapshots of the intracellular composition possible. The technique
25 in the illustrated embodiment is described in connection with adherent cells, but it is also applicable to nonadherent cells, which are either loaded physically into inlet 48 or held by laser microbeam optical tweezers (not shown). The improved rapidity of this technique, the absence of cell trauma prior to lysis and its application to both adherent and nonadherent single cells
30 will expand the role of microanalytical single cell measurements. The applications of the present invention will be described in greater detail below.

Consider now a specific illustrated embodiment starting with the reagents used. Fluorescein diacetate (mixed isomers), fluorescein (fluorescein free acid), Oregon Green 488 carboxylic acid diacetate 6-isomer (Oregon Green diacetate), and Oregon Green 488 carboxylic acid 6-isomer (Oregon Green free acid), was prepared by and obtained from Molecular Probes of Eugene, Oregon. A buffer solution, which will be referenced here as buffer A is a physiologic extracellular buffer used as the electrolyte medium in capillary 22 between proximate end 26 and cover slip 36/ Buffer A is composed of 135 mM of NaCl, 5 mM of KCl, 10 mM of Hepes, 2 mM of MgCl₂, 2 mM of CaCl₂ and adjusted to a pH of 7.4 with NaOH from reagents purchased from Fisher Scientific of Pittsburgh, Pennsylvania.

Rat basophilic leukemia (RBL) cells, a tumor mast cell line, were used as the model system for the illustrated embodiment. The cells were grown at 37(C and 5% CO₂ in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and L-glutamine (584 mg / L). Penicillin (100 units per ml) and streptomycin (100 (g/ ml), were added to the media to inhibit bacterial growth. Cell culture materials were obtained from Gibco BRL of Gaithersburg, Md. The cells were grown in a cell chamber and made by using Sylgard from Dow Corning of Midland, Michigan, to attach a Teflon O ring of 15/16 inch outer diameter to a 25 mm diameter round number 1 glass cover slip. Cell attachment to the glass surface was enhanced by coating the cover slip with Cell-Tak made by Becton Dickinson of Bedford, Massachusetts, prior to adding the cells to the cell chamber. Prior to use, the cells were allowed to grow in the supplemented medium for 12 to 24 hours after plating in the cell chamber. The cells were plated at concentrations determined empirically to produce approximately one cell in a 63x field of view at the time the laser exposure was made.

The cells were loaded with fluorescein diacetate and/or Oregon Green diacetate. These cell permeant compounds are not fluorescent until they are hydrolyzed by ubiquitous intracellular esterases after the compounds pass into the cell. Once the fluorescent free acid is formed, it is no longer cell permeant and remains trapped within the cell although the cells do transport

the dye out over time via anionic transporters. For the single cell experiment cells grown in DMEM in a cell chamber were washed once in Buffer A.

Solutions of fluorescein (20 nM) and/or Oregon Green (500 nM) diacetates were made in 400 l buffer A plus 10 mM glucose immediately prior to use

5 and added to the cell chamber after removing the wash buffer. The cells were incubated at room temperature in the dark for 30 minutes to load the fluorescent compounds into the cells. The cells were then washed five times in buffer A and used within 10 to 15 minutes thereafter. The amount of dye loaded into each cell by this method varies to some degree due to cell-to-cell

10 differences in the hydrolysis of the diacetate and due to loss of the free acid from the intracellular space. Estimates of the moles of fluorescent marker obtained from each cell were made by comparison of fluorescent peak areas with comparison to standards in electropherograms. Fresh buffer A, 10 ml per minute, was continually exchanged in the cell chamber during the course of the experiment in order to remove the dye expelled from the cells into the surrounding buffer. In the absence of the flow system, dye accumulated in the extracellular buffer over time, increasing the fluorescence baseline.

15 The temporal resolution of the biological measurements used for a single cell analysis is determined by the time between initiation of the sampling process and termination of cellular reactions involving the molecule(s) of interest.

20 Termination of these reactions is achieved by separation of the reactants usually by mixing, diffusion and/or electrophoresis or by inactivation of the reactants, typically by denaturation of the proteins. Minimizing the time required for cell lysis and initiation of analysis, such as by capillary

25 electrophoresis, enhances the temporal resolution of single cell biochemical analyses. As discussed below, cell lysis in msec time scales followed by loading of cellular contents into a capillary for separation detection dramatically improves the temporal resolution of capillary electrophoresis based chemical measurements of single cells.

30 To determine the characteristics of a cell lysis by production of a shock wave, a single pulsed laser microbeam with an energy content in the range of 20-100 J was employed as described above to lyse the RBL cells while the

video camera recorded the bright field image every 33 msec as depicted in the sequence of depictions in Fig. 2A-B. Fig. 2A is a photodepiction of an RBL cell prior to lysis and Fig. 2B at the moment of lysis showing a reflection of the laser beam immediately to the right of a cell to be lysed. Thirty-three milliseconds thereafter as shown in Fig. 2C, the cell has been completely disrupted, leaving only fragments of the cell membrane. Substantially, all the cell contents are released within thirty-three msec subsequent to lysis and there is no substantial difference between the condition of the field of view at 2C and five seconds later as shown in Fig. 2D. Direct, localized attenuation of a portion of the plasma membrane is performed by focusing the laser pulse at lower energy densities directly on the cell membrane. In experiments described here, complete lysis is achieved without direct exposure of the cell to the laser beam. In this way, neither photodestruction nor photobleaching of intracellular species occurs. In the lysis of the cell the beam was positioned 20-30 microns to one side of cell 46, as depicted in Fig. 2B. The shock wave was generated by focusing the pulsed laser beam with a 0.3 to 0.5 micron diameter at its waist 50 within coverslip 36 just below the coverslip-to-buffer interface. The same shock wave effect could also be obtained by using other microscope objectives ranging in magnification from 40X to 100X.

RBL cells contain histamine and serotonin within secretory granules. These granules appear as small particles released upon cell lysis that move by Brownian motion. Using lower energies, 10-20 (J, to generate the shock wave, the cell membrane appears to be permeabilized by the shock wave.

These cells become spherical after the shock wave has passed through the cells and the cellular contents, namely the granules, leak out of the cell over 20-30 video frames. At higher energies above 20 (J, the membrane is completely disrupted, leaving only remnants of the membrane present in the first video frame as shown in Fig. 2C after impingement of the shock wave.

The actual time period over which lysis occurs is much shorter than 33 msec, and possibly as brief as 25 - 30 nsec. 33 msec is chosen here only as the lowest time resolution of the video analysis which was limited by the 30 frame

per second video rate. At these higher energies the secretory granules are again seen in the immediate vicinity, that is within about 10 microns of the remnants of the plasma membrane. The fact that the secretory granules remain localized suggests that only limited dilution of cellular contents

5 occurred at the time of lysis. By increasing the beam energy over the range of 20-100 (J, cells could be lysed at greater distances from its focal point. This laser-based method of cell lysis allows for local attenuation of a cell membrane in such a manner that the degree and field of cell lysis can be selectively controlled. This controllability makes it possible to obtain
10 subcellular measurements of small cells by lysing a portion of the cell, i.e. lysis of a cell extension or rupture of the nucleus where the genetic material resides.

In each case of cell lysis, a small divot appears in glass coverslip 36 at the point where the laser beam was focused. In some instances, a bubble with a
15 diameter of less than 5-10 microns appeared in the first frame following the laser pulse at the intersection of the laser beam and the glass buffer interface. The bubble was absent in subsequent frames. In other experiments, the circular wave could be seen emanating from the location of the laser beam in a video frame coincident with the pulse. Thirty-three
20 milliseconds later the cell was obliterated, and the wave had disappeared as depicted in the series of frame shots shown in Fig. 3A, 3B and 3C. The divot, bubble and wave further support the contention that a plasma is formed on cover slip 36 thereby producing cavitation and a shock wave.

To demonstrate that the contents of an adherent RBL cell are then loaded
25 into capillary 22 after lysis, a single RBL cell 46 containing fluorescein was positioned below in proximate inlet 48 of lumen 52 of capillary 22. Inlet 48 of capillary 22 was approximately 15-25 microns above cell 46, although capillary 22 could be positioned flush with cover slip 36 so the cell actually sat within the entrance to lumen 52 if desired. Cell 46 was lysed as described
30 above and electrophoresis was initiated upon cell lysis. Analysis of the video images show the cellular contents had disappeared within 33 msec after the lysis pulse as shown in Fig. 3C. The cellular contents had thus been loaded

within capillary 22 in less than 33 msec. In some experiments the plasma membrane remnants remain attached to glass-coverslip 36 while in other experiments the remnants were also loaded into capillary 22. The current and migration times of fluorophores from the cells were the same in both cases. In these experiments, the cellular contents were loaded into capillary 22 by gravity siphon flow with some later contribution from electrophoresis. Although not utilized in these experiments, by triggering the capillary electrophoretic power supply with the trigger pulse of the laser, electrophoresis could be initiated coincidentally with the lysis pulse. The use of a capillary with charged lumenal walls allows the use of electroosmotic flow, and loading the cell's contents by the gravity siphon flow could be eliminated, if desired. Some loading also occurs by virtue of the laser induced shockwave.

Turn now and consider the electrophoretic traces shown in Fig. 4. All electrophoretic runs were performed on the same day using the same capillary, namely a 100 cm capillary having a 50 micron inner diameter, a 360 micron outer diameter, a detection window 85 cm from the inlet with an applied voltage of 15 kV at 60 microamp. The amount of fluorophores was determined by comparison with standards. Trace A of Fig. 4 shows the detection of fluorescein which was loaded into the cell and which was detected after cell lysis and electrophoresis was performed. A single peak containing fluorescein is depicted. Trace B of Fig. 4 is an electrophoretic trace from a single cell loaded with Oregon Green. The four reproducible peaks of Oregon Green diacetate are clearly illustrated. The fluorophore in the largest peak corresponds to free acid. Trace C of Fig. 4 is the trace from a single cell loaded with both fluorescein and Oregon Green showing the superposition of traces A and B. Trace D of Fig. 4 shows the detection of Oregon Green and fluorescein in the free acid standards.

Trace A of Fig. 4 shows a single peak with the same migration time as that of fluorescein in the buffer in the electropherogram of trace D, thereby confirming that the cell's contents were in fact loaded into capillary 22. Electrophoretic traces (not shown) of cells not containing a fluorescent

marker showed no peaks. Likewise, when inlet 48 of capillary 22 was placed at a distance just beyond the field of view from a fluorescein containing cell, the fluorescent baseline of the electropherogram (not shown) remained flat after cell lysis, indicating failure to load the cell contents within capillary 22.

5 In some experiments more than one cell was present in the vicinity of inlet 48. By employing higher laser energies to lyse the cell immediately below inlet 48, multiple cells could be lysed. Peaks corresponding to these adjacent cells could then be seen separately in the electropherogram. It is anticipated, therefore, that in most applications cell lysis will be controlled or the density of
10 the cell population maintained so that only a single cell's contents are loaded. However, this is not to necessarily exclude applications where lysis of multiple cells by the same laser pulse might for some reason be desired.

A series of experiments were conducted in which RBL cells were loaded with fluorescein or Oregon Green alone or in combination. A single cell containing
15 the fluorophore was then lysed and its contents loaded into capillary 22 and electrophoresis performed as described above. Traces A - C of Fig. 4 show representative traces for these experiments. Interestingly, while

electrophoresis traces of cells loaded with fluorescein diacetate alone demonstrated a single peak, cells with Oregon Green diacetate alone
20 consistently demonstrated three distinct peaks of nearly equal magnitude and a smaller fourth peak. Migration time of the initial Oregon Green peak was identical to that Oregon Green free acid in buffer. The subsequent peaks were not seen in the electrophoretic traces of the Oregon Green free acid standard. No fluorescent peaks were identified when Oregon Green

25 diacetate (3.8×10^{-15} mol) was electrophoresced in buffer. The two additional major peaks in the Oregon Green traces may be forms of partially hydrolyzed Oregon Green diacetate or the additional peaks may be impurities that are concentrated within the cell. As a result of the occurrence of these additional peaks in the Oregon Green signal, it is possible to demonstrate the
30 resolution of five distinct species from a single cell as shown in trace C of Fig.

4.

As the demonstration of feasibility of serial analysis of the contents of two cells using the technique of the invention, capillary 22 was loaded with two cells lysed in succession. Two cells were contained in the same cell chamber and loaded with fluorescein and Oregon Green diacetates in a similar manner. A single loaded cell below a proximate inlet 48 of capillary 22 was lysed and its contents introduced into capillary 22 by gravity siphon flow. The second cell was then centered below inlet 48 during which time buffer continued to flow into the capillary by gravity. The second cell was lysed and electrophoresis was performed immediately following lysis. Thus, the contents of the second cell were loaded by a combination of gravity, electrophoresis and possibly momentum created by the laser induced shock wave. The electropherogram of Fig. 5 shows the analytes from each cell as distinct peaks. Fluorescent signals from the first cell denoted by subscript "1" are lower than those for the second cell denoted by subscript "2". This difference in signal intensity is most likely due to the biologic diversity of cellular uptake and differences in the metabolism of the two cells.

Many alterations and modifications may be made by those having ordinary skill in the art without departing from the spirit and scope of the invention. Therefore, it must be understood that the illustrated embodiment has been set forth only for the purposes of example and that it should not be taken as limiting the invention as defined by the following claims.

Although there is no intended limit to the applications to which the invention can be applied, it is expressly contemplated that such modifications within the skill of the art as may be needed to use the invention in diagnostic biopsies including DNA analysis using polymerase chain reactions or other biochemical methods in both human and animal medicine are included within the scope of the claims. The use of the invention in combination with conventional pathology techniques to provide confirmation or automation of pathological identification of diseased states is included. Significant application of the invention in the selection and analysis of hybridoma cells is specifically intended. The technique has clear application to drug efficacy testing both in vitro and in vivo and to gene sequencing and therapy. As

mapping the human genome proceeds, the invention will be a useful tool in detecting and diagnosing variances in gene expression, RNA, and protein function and in correcting or mitigating these deficiencies. The invention is broadly applicable to other species and genomes as well.

- 5 The use of any type of mechanism beyond siphon, electrophoretic, electroosmotic and shock wave forces to collect the cell contents into a pipette is expressly included in the scope of the invention. Analysis by polymerase chain reactions or any other microanalytical techniques now known or later devised is considered as equally applicable as the described
- 10 electrophoretic laser induced fluorescence. The controllability of cell selection will allow new applications where analysis of a cell marked by chemical or other techniques is performed. Whereas the illustrated embodiment describes a fluid buffer in which the cell is cultured, lysed and its contents then extracted in a flow, it is expressly contemplated that the techniques can
- 15 be modified from those illustrated to include any type of medium including solid or semisolid matrices or tissues into which collection and fiber optics can be inserted. For example biologic matrices mounted on silicon or solid substrates can be combined with the claimed technique to provide partial or completely lysed cell analysis in combination with electrochemical
- 20 measurement of the cells and cell contents and of tissue sections. The analysis of both endogenous cellular components such as DNA, RNA, proteins, small organic molecules such as second messengers and others as well as exogenous or artificial components introduced into the cell, such as enzyme substrates, molecules with bromodeoxyuridine or other chemical
- 25 markers that can be used to identify other molecules, are specifically anticipated.

The words used in this specification to describe the invention and its various embodiments are to be understood not only in the sense of their commonly defined meanings, but to include by special definition in this

30 specification structure, material or acts beyond the scope of the commonly defined meanings. Thus if an element can be understood in the context of this specification as including more than one meaning, then its use in a claim

must be understood as being generic to all possible meanings supported by the specification and by the word itself.

The definitions of the words or elements of the following claims are, therefore, defined in this specification to include not only the combination of
5 elements which are literally set forth, but all equivalent structure, material or acts for performing substantially the same function in substantially the same way to obtain substantially the same result. In this sense it is therefore contemplated that an equivalent substitution of two or more elements may be made for any one of the elements in the claims below or that a single element
10 may be substituted for two or more elements in a claim.

Insubstantial changes from the claimed subject matter as viewed by a person with ordinary skill in the art, now known or later devised, are expressly contemplated as being equivalently within the scope of the claims. Therefore, obvious substitutions now or later known to one with ordinary skill in the art
15 are defined to be within the scope of the defined elements.

The claims are thus to be understood to include what is specifically illustrated and described above, what is conceptionally equivalent, what can be obviously substituted and also what essentially incorporates the essential idea of the invention.

We claim:

1. A method for lysing and analysis of the contents of a selected cell or cellular component thereof comprising:
controllably selecting at least one of a plurality of cells or cellular component
5 thereof, in a medium;
disrupting said at least one selected cell or cellular component thereof with a laser generated shock wave in said medium;
collecting at least a portion of said contents of said cell or cellular component thereof in an analysis device; and
10 analyzing said collected contents.
2. The method of claim 1 wherein said portion of said contents of said cell or cellular component thereof is collected within one second of lysis or less of said cell.
3. The method of claim 1 wherein said portion of said contents of said cell
15 or cellular component thereof is collected within 33 msec or less of lysis of said cell.
4. The method of claim 1 wherein said portion of said contents of said cell or cellular component thereof is collected within a 1 - 10 microseconds of lysis of said cell.
- 20 5. The method of claim 1 wherein controllably selecting at least one of a plurality of cells or cellular component thereof comprises identifying and relative positioning said selected cell or cellular component thereof.

6. The method of claim 1 wherein collecting at least a portion of said contents of said cell or cellular component thereof comprises stopping reactions of biochemical reactants disrupted from said selected cell or cellular component thereof to permit subsequent analysis of said biochemical
5 reactants in the state which existed approximately at the time of disruption.

7. The method of claim 5 where controllably positioning said selected cell or cellular component thereof in said medium comprises adhering said cell or cellular component thereof to a substrate disposed at least adjacent to said medium.

10 8. The method of claim 5 where said cell or cellular component is free floating and where controllably positioning said selected cell or cellular component thereof in said medium comprising temporarily holding said cell or cellular component thereof in a position in said medium by laser microbeam optical tweezers.

15 9. The method of claim 5 where said cell or cellular component is free floating and where controllably positioning said selected cell or cellular component thereof in said medium comprises temporarily holding said cell or cellular component thereof in a position in said medium by adhesion of a mechanical micromanipulator-held pipette or other device to said cell or
20 cellular component thereof.

10. The method of claim 5 where said cell or cellular component is free floating and where controllably positioning said selected cell or cellular component thereof in said medium comprising positioning said cell or cellular component thereof in a confined enclosure.

11. The method of claim 10 where said cell or cellular component is free floating and where positioning said cell or cellular component thereof in said confined enclosure comprises positioning said cell or cellular component thereof in an inlet to said analysis device.

5 12. The method of claim 1 where collecting at least a portion of said contents of said disrupted cell or cellular component thereof in said analysis device collects said cell or cellular component thereof in an electrophoretic column.

10 13. The method of claim 2 where collecting at least a portion of said contents of said disrupted cell or cellular component thereof in said analysis device collects said cell or cellular component thereof in an electrophoretic column.

15 14. The method of claim 13 where analyzing said collected contents comprising electrophoretically analyzing said contents using laser induced fluorescence.

20 15. The method of claim 1 where disrupting said selected cell or cellular component with a laser generated shock wave in said medium comprises focusing a pulsed laser beam at a position proximate to said cell or cellular component thereof, without focusing on said cell or cellular component thereof, and generating said shock wave.

16. The method of claim 1 where disrupting said selected cell or cellular component thereof with a laser generated shock wave in said medium comprises focusing a pulsed laser beam directly in or on said cell or cellular component thereof and generating said shock wave.

17. The method of claim 16 where disrupting said selected cell or cellular component thereof by focusing a pulsed laser beam directly in or on said cell or cellular component thereof defines an opening in said cell or cellular component thereof to lyse only cytoplasmic contents therefrom.

5 18. The method of claim 1 where collecting at least a portion of said contents of said disrupted cell or cellular component thereof in said analysis device is by means of fluid flow of said medium.

19. The method of claim 18 where collecting at least a portion of said contents of said disrupted cell or cellular component thereof in said analysis
10 device is by means of siphon fluid flow of said medium.

20. The method of claim 1 where collecting at least a portion of said contents of said disrupted cell or cellular component thereof in said analysis device is by means of electrophoresis through said medium.

21. The method of claim 18 where collecting at least a portion of said
15 contents of said disrupted cell or cellular component thereof in said analysis device is by means of force from said shock wave impacted to said contents.

22. The method of claim 18 where collecting at least a portion of said contents of said disrupted cell or cellular component thereof in said analysis device is by means of electroosmotic fluid flow.

23. An apparatus for lysing and analysis of the contents of one of a plurality of cells or cellular components thereof comprising:
a cell selector to controllably select at least one of said cells or cellular component thereof;
- 5 a laser for generating a pulse to lyse said at least one selected cell or cellular component thereof;
an analysis device for analyzing said contents; and
a collector to capture at least a portion of said contents of said lysed cell or cellular component thereof in said analysis device.
- 10 24. The apparatus of claim 23 wherein said collector delivers at least said portion of said contents of said lysed cell or cellular component to said analysis device within one second of lysis of said cell or cellular component.
25. The apparatus of claim 23 wherein said collector delivers at least said portion of said contents of said lysed cell or cellular component to said
- 15 analysis device within 33 msec of lysis of said cell or cellular component.
26. The apparatus of claim 23 wherein said collector delivers at least said portion of said contents of said lysed cell or cellular component to said analysis device within 1 - 10 microseconds of lysis of said cell or cellular component.
- 20 27. The apparatus of claim 23 wherein said analysis device comprises a means for performing polymerase chain reactions on said contents, a means of separating the product of said polymerase chain reactions, and a means for detecting separated molecules.
28. The apparatus of claim 23 wherein said cell or cellular component is
- 25 identified by said selector and wherein said laser generates a shock wave in a medium proximate to said identified cell or cellular component.

29. The apparatus of claim 23 wherein said collector delivers said portion by flowing a medium past said cell or cellular component during lysis into said analysis device.

30. The apparatus of claim 23 wherein said collector delivers said portion by providing electrophoresis in a medium between said analysis device and said cell or cellular component during lysis thereof.

31. The apparatus of claim 23 wherein said collector delivers said portion by providing an electroosmotic flow in a medium between said analysis device and said cell or cellular component during lysis thereof.

32. The apparatus of claim 23 wherein said collector delivers said portion by providing force from a shock wave in a medium to move said cell or cellular component into said analysis device during lysis of said cell or cellular component.

33. A method for fast lysing and analysis of the contents of one of a plurality of cells or cellular components thereof comprising:
controllably selecting at least one of said plurality of cells or cellular components thereof by relative placement of said selected cell or component adjacent to an inlet orifice of a pipette;
lysing said selected cell or cellular component thereof with a laser generated pulse;
collecting at least a portion of said contents of said lysed cell or cellular component thereof in said pipette within one second of lysis for subsequent analysis; and
stopping further substantial biologic reactions in said contents after lysis.

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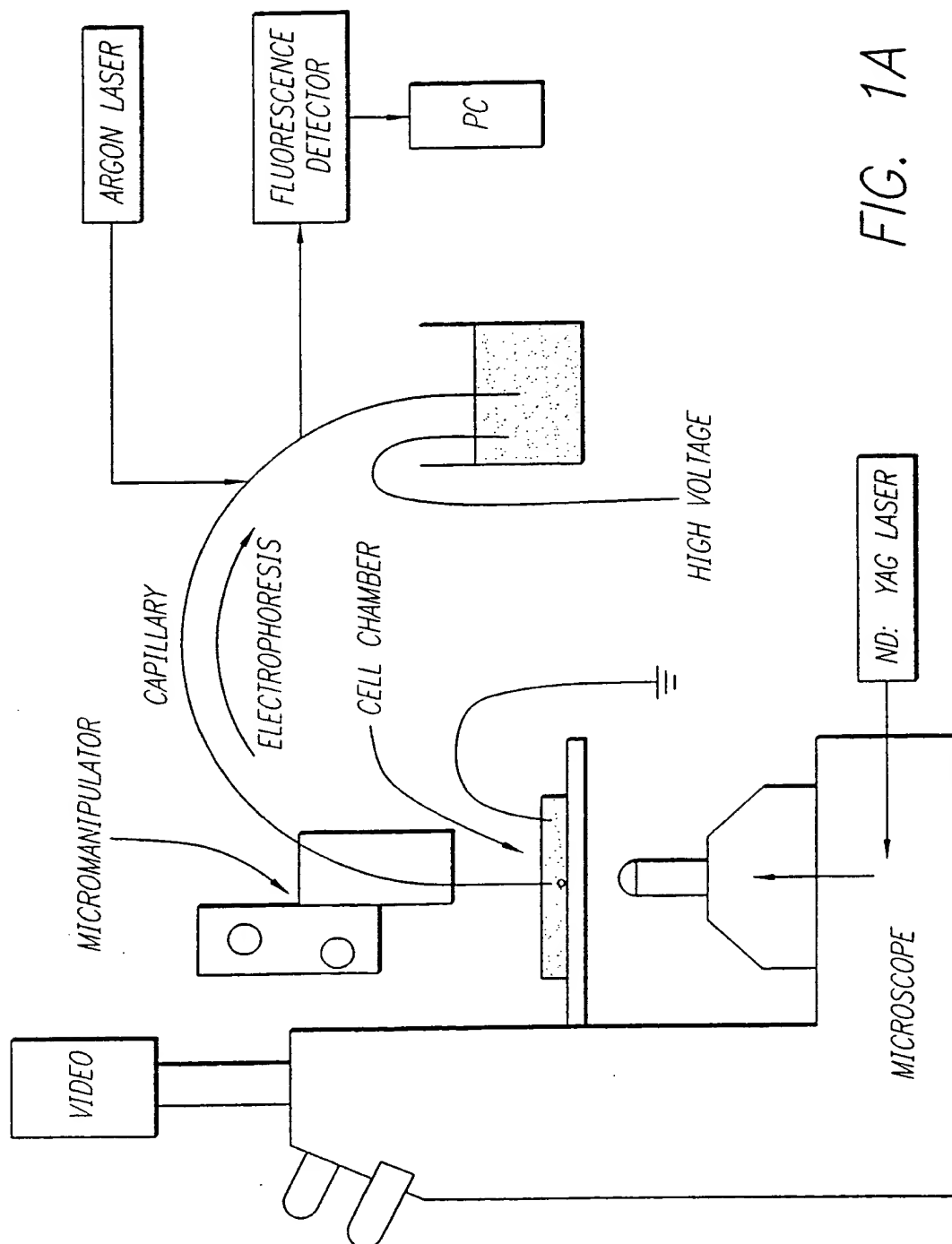


FIG. 1A

FIG. 1C

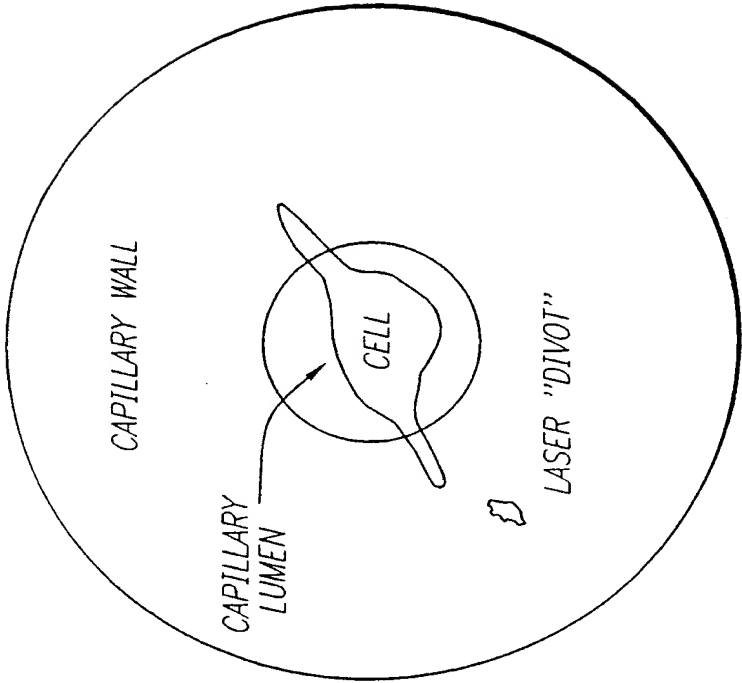
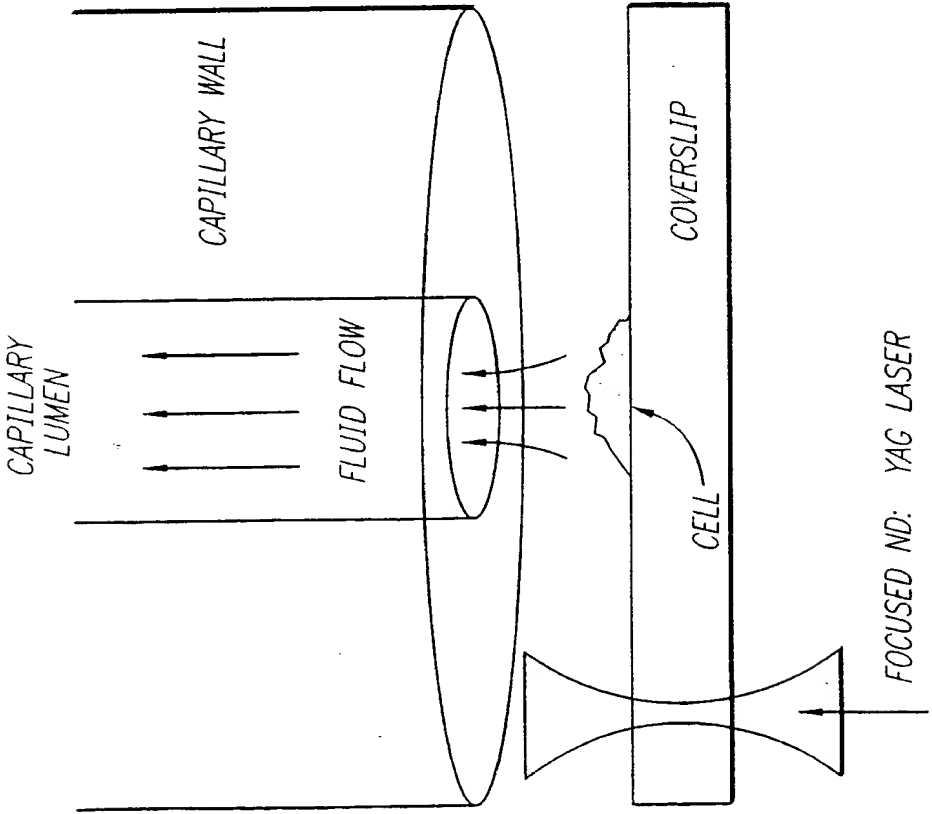
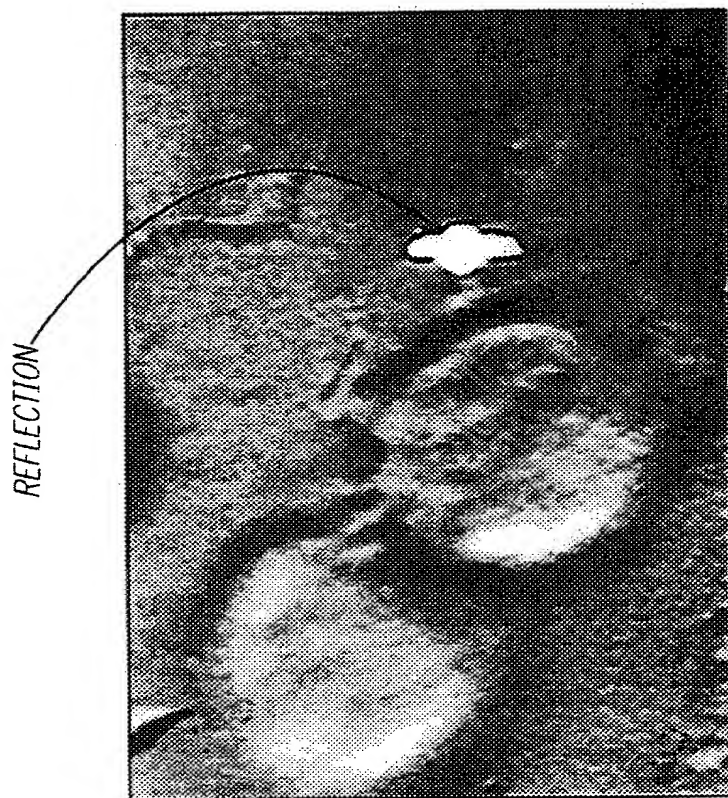


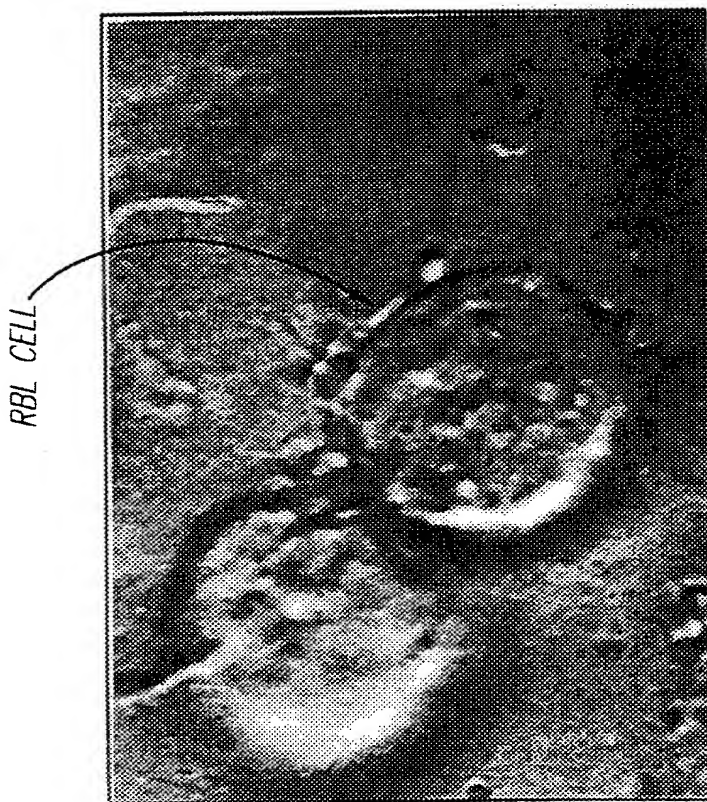
FIG. 1B



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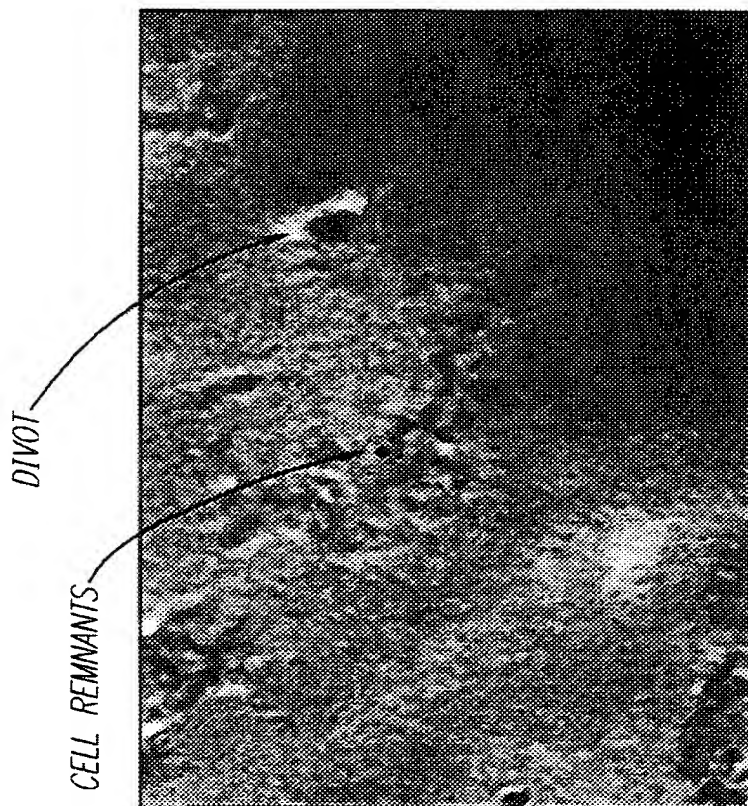


LYSIS
FIG. 2B



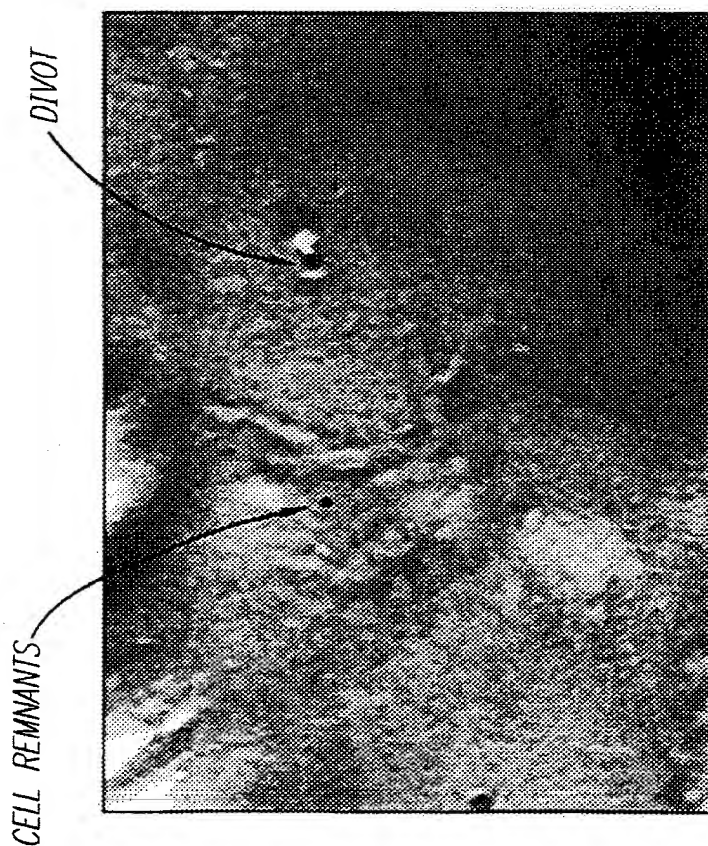
PRE-LYSIS
FIG. 2A

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POST-LYSIS, 5 SEC

FIG. 2C



POST-LYSIS, 33 MSEC

FIG. 2C

SUBSTITUTE SHEET (RULE 26)

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FIG. 3A

PRE-LYSIS

CAPILLARY LUMEN

RBL CELL

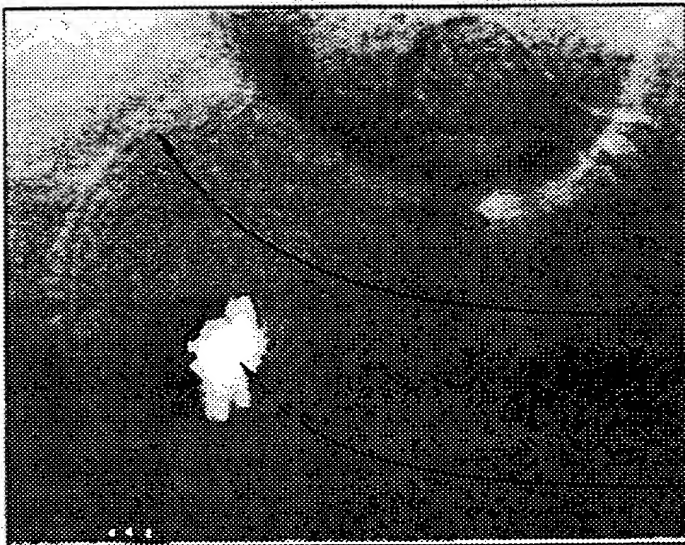


FIG. 3B

LYSIS

CIRCULAR WAVE

REFLECTION

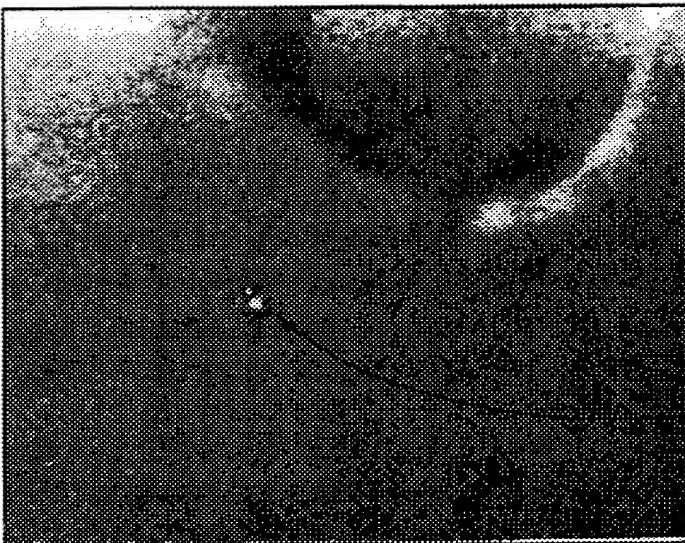
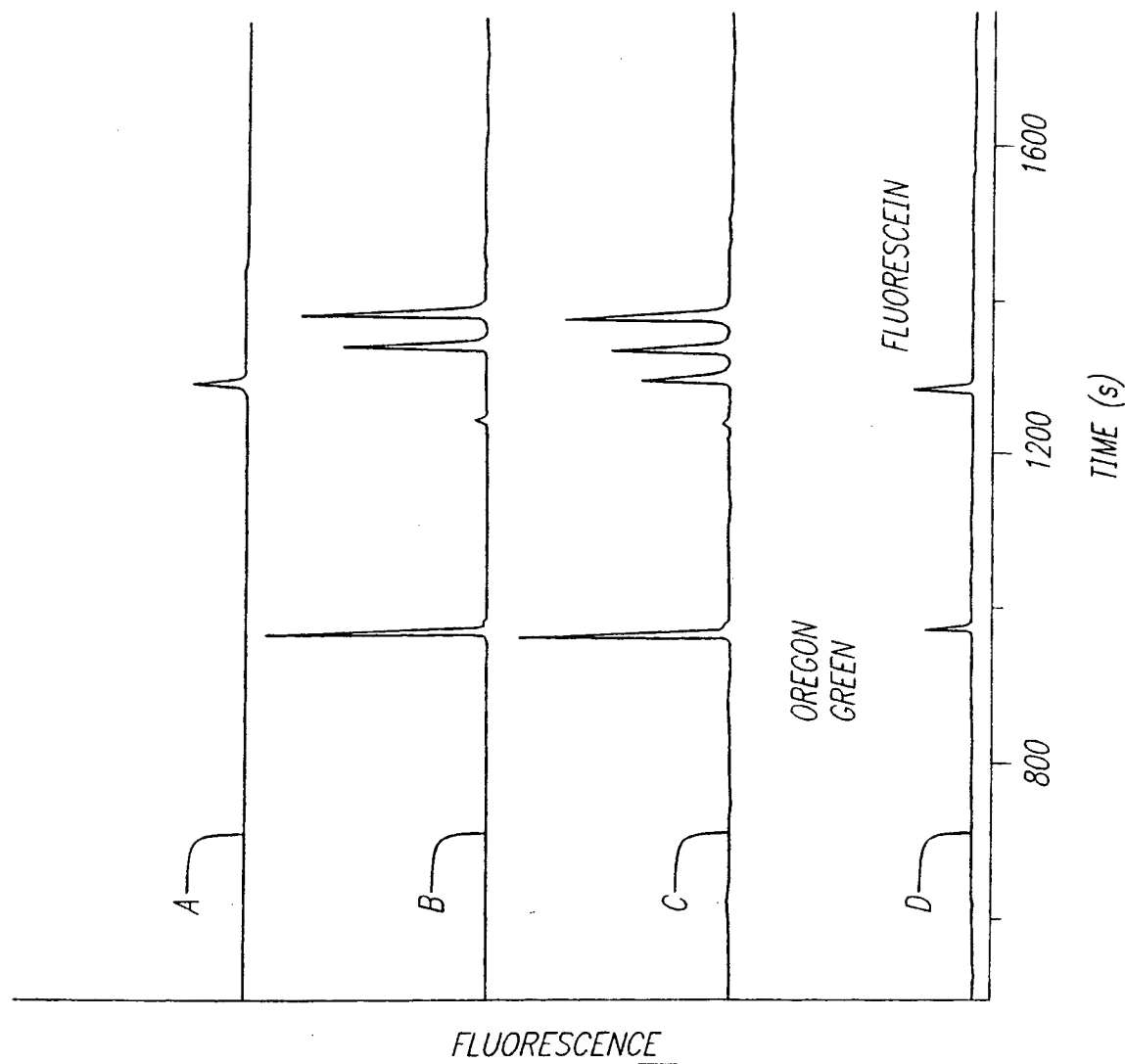


FIG. 3C

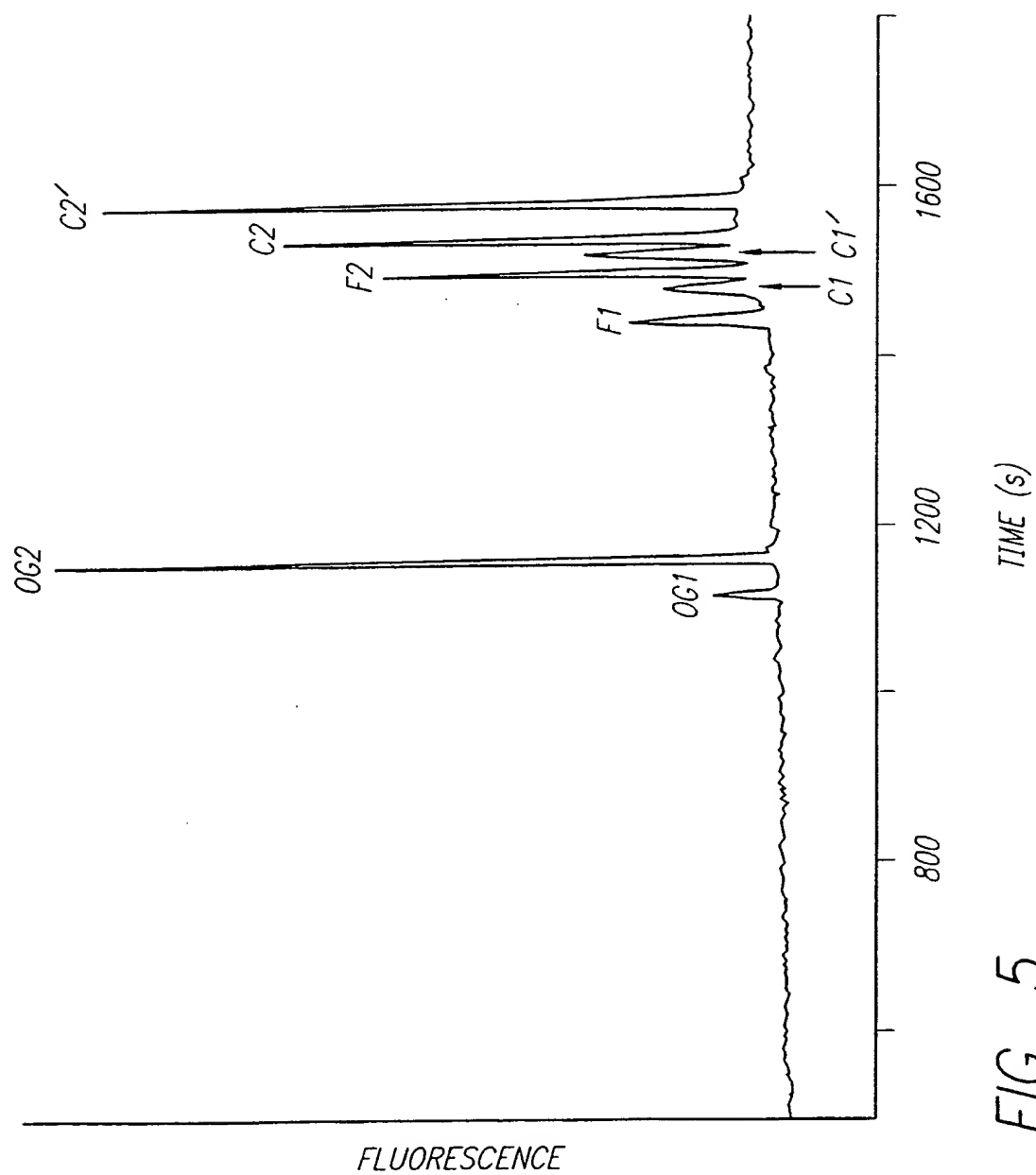
POST-LYSIS, 33 msec

DIVOT

FIG. 4



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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04595

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : GO1N 27/26, 33/48

US CL : Please See Extra Sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/63, 164, 172; 422/82.05, 82.08; 435/4, 29, 30; 288.7 204/400, 403, 450, 451, 452, 453, 600, 601, 603, 604

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN/CA, BIOSIS, MEDLINE

search terms: cell, lysis, laser, shock wave

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	SIMS, CHRISTOPHER E. et al. Laser-Micropipet Combination for Single-Cell Analysis. Analytical Chemistry. 01 November 1998, Vol. 70, No. 21, pages 4570-4577, especially pages 4572-4573.	1-33
A	LUZZI, VERONICA et al. Localized Sampling of Cytoplasm from Xenopus Oocytes for Capillary Electrophoresis. Analytical Chemistry. 01 December 1997, Vol. 69, No. 23, pages 4761-4767.	1-33
A	DOUKI, TINA et al. Stress-Wave-Induced Injury to Retinal Pigment Epithelium Cells In Vitro. Lasers in Surgery and Medicine. 1996, Vol. 19, pages 249-259.	1-33

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

20 MAY 1999

Date of mailing of the international search report

28 MAY 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04595

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEE, SHUN et al. Alteration of Cell Membrane by Stress Waves In Vitro. Ultrasound in Medicine and Biology. 1996, Vol. 22, No. 9, pages 1285-1293.	1-33
A	TONG, WEI et al. Monitoring Single-Cell Pharmacokinetics by Capillary Electrophoresis and Laser-Induced Native Fluorescence. Journal of Chromatography. 1997, Vol. 689, pages 321-325.	1-33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04595

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

436/63, 164, 172; 422/82.05, 82.08; 435/4, 29, 30; 204/451, 452, 453, 601, 603, 604

role in the identification of potential new targets. Proteomics has become indispensable in relating structure and function of protein targets in order to predict drug interactions. However, the next level of biological complexity is the cell. Therefore, there is a need to acquire, manage and search multi-dimensional information from cells. Secondly, there is a need for higher throughput tools. Automation is a key to improving productivity as has already been demonstrated in DNA sequencing and high throughput primary screening. The instant invention provides for automated systems that extract multiple parameter information from cells that meet the need for higher throughput tools. The instant invention also provides for miniaturizing the methods, thereby allowing increased throughput, while decreasing the volumes of reagents and test compounds required in each assay.

Radioactivity has been the dominant read-out in early drug discovery assays. However, the need for more information, higher throughput and miniaturization has caused a shift towards using fluorescence detection. Fluorescence-based reagents can yield more powerful, multiple parameter assays that are higher in throughput and information content and require lower volumes of reagents and test compounds. Fluorescence is also safer and less expensive than radioactivity-based methods.

Screening of cells treated with dyes and fluorescent reagents is well known in the art. There is a considerable body of literature related to genetic engineering of cells to produce fluorescent proteins, such as modified green fluorescent protein (GFP), as a reporter molecule. Some properties of wild-type GFP are disclosed by Morise et al. (*Biochemistry* 13 (1974), p. 2656-2662), and Ward et al. (*Photochem. Photobiol.* 31 (1980), p. 611-615). The GFP of the jellyfish *Aequorea victoria* has an excitation maximum at 395 nm and an emission maximum at 510 nm, and does not require an exogenous factor for fluorescence activity. Uses for GFP disclosed in the literature are widespread and include the study of gene expression and protein localization (Chalfie et al., *Science* 263 (1994), p. 12501-12504)), as a tool for visualizing subcellular organelles (Rizzuto et al., *Curr. Biology* 5 (1995), p. 635-642)), visualization of protein transport along the secretory pathway (Kaether and Gerdes, *FEBS Letters* 369 (1995), p. 267-271)), expression in plant cells (Hu and Cheng, *FEBS Letters* 369 (1995), p. 331-334)) and *Drosophila* embryos (Davis et al., *Dev. Biology* 170 (1995), p. 726-729)), and as a reporter molecule fused to another protein of interest (U. S. Patent

5,491,084). Similarly, WO96/23898 relates to methods of detecting biologically active substances affecting intracellular processes by utilizing a GFP construct having a protein kinase activation site. This patent, and all other patents referenced in this application are incorporated by reference in their entirety.

5 Numerous references are related to GFP proteins in biological systems. For example, WO 96/09598 describes a system for isolating cells of interest utilizing the expression of a GFP like protein. WO 96/27675 describes the expression of GFP in plants. WO 95/21191 describes modified GFP protein expressed in transformed organisms to detect mutagenesis. U. S. Patents 5,401,629 and 5,436,128 describe
10 assays and compositions for detecting and evaluating the intracellular transduction of an extracellular signal using recombinant cells that express cell surface receptors and contain reporter gene constructs that include transcriptional regulatory elements that are responsive to the activity of cell surface receptors.

Performing a screen on many thousands of compounds requires parallel
15 handling and processing of many compounds and assay component reagents. Standard high throughput screens ("HTS") use mixtures of compounds and biological reagents along with some indicator compound loaded into arrays of wells in standard microtiter plates with 96 or 384 wells. The signal measured from each well, either fluorescence emission, optical density, or radioactivity, integrates the signal from all the material in
20 the well giving an overall population average of all the molecules in the well.

Science Applications International Corporation (SAIC) 130 Fifth Avenue, Seattle, WA. 98109) describes an imaging plate reader. This system uses a CCD camera to image the whole area of a 96 well plate. The image is analyzed to calculate the total fluorescence per well for all the material in the well.

25 Molecular Devices, Inc. (Sunnyvale, CA) describes a system (FLIPR) which uses low angle laser scanning illumination and a mask to selectively excite fluorescence within approximately 200 microns of the bottoms of the wells in standard 96 well plates in order to reduce background when imaging cell monolayers. This system uses a CCD camera to image the whole area of the plate bottom. Although this system
30 measures signals originating from a cell monolayer at the bottom of the well, the signal measured is averaged over the area of the well and is therefore still considered a measurement of the average response of a population of cells. The image is analyzed to

calculate the total fluorescence per well for cell-based assays. Fluid delivery devices have also been incorporated into cell based screening systems, such as the FLIPR system, in order to initiate a response, which is then observed as a whole well population average response using a macro-imaging system.

5 In contrast to high throughput screens, various high-content screens ("HCS") have been developed to address the need for more detailed information about the temporal-spatial dynamics of cell constituents and processes. High-content screens automate the extraction of multicolor fluorescence information derived from specific fluorescence-based reagents incorporated into cells (Giuliano and Taylor (1995), *Curr.*
10 *Op. Cell Biol.* 7:4; Giuliano et al. (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405). Cells are analyzed using an optical system that can measure spatial, as well as temporal dynamics. (Farkas et al. (1993) *Ann. Rev. Physiol.* 55:785; Giuliano et al. (1990) In *Optical Microscopy for Biology*. B. Herman and K. Jacobson (eds.), pp. 543-557. Wiley-Liss, New York; Hahn et al (1992) *Nature* 359:736; Waggoner et al. (1996)
15 *Hum. Pathol.* 27:494). The concept is to treat each cell as a "well" that has spatial and temporal information on the activities of the labeled constituents.

The types of biochemical and molecular information now accessible through fluorescence-based reagents applied to cells include ion concentrations, membrane potential, specific translocations, enzyme activities, gene expression, as well as the
20 presence, amounts and patterns of metabolites, proteins, lipids, carbohydrates, and nucleic acid sequences (DeBiasio et al., (1996) *Mol. Biol. Cell.* 7:1259; Giuliano et al., (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405; Heim and Tsien, (1996) *Curr. Biol.* 6:178).

High-content screens can be performed on either fixed cells, using fluorescently
25 labeled antibodies, biological ligands, and/or nucleic acid hybridization probes, or live cells using multicolor fluorescent indicators and "biosensors." The choice of fixed or live cell screens depends on the specific cell-based assay required.

Fixed cell assays are the simplest, since an array of initially living cells in a microtiter plate format can be treated with various compounds and doses being tested,
30 then the cells can be fixed, labeled with specific reagents, and measured. No environmental control of the cells is required after fixation. Spatial information is acquired, but only at one time point. The availability of thousands of antibodies,

ligands and nucleic acid hybridization probes that can be applied to cells makes this an attractive approach for many types of cell-based screens. The fixation and labeling steps can be automated, allowing efficient processing of assays.

Live cell assays are more sophisticated and powerful, since an array of living
5 cells containing the desired reagents can be screened over time, as well as space. Environmental control of the cells (temperature, humidity, and carbon dioxide) is required during measurement, since the physiological health of the cells must be maintained for multiple fluorescence measurements over time. There is a growing list
10 of fluorescent physiological indicators and "biosensors" that can report changes in biochemical and molecular activities within cells (Giuliano et al., (1995) *Ann. Rev. Biophys. Biomol. Struct.* 24:405; Hahn et al., (1993) In *Fluorescent and Luminescent Probes for Biological Activity*. W.T. Mason, (ed.), pp. 349-359, Academic Press, San Diego).

The availability and use of fluorescence-based reagents has helped to advance
15 the development of both fixed and live cell high-content screens. Advances in instrumentation to automatically extract multicolor, high-content information has recently made it possible to develop HCS into an automated tool. An article by Taylor, et al. (*American Scientist* 80 (1992), p. 322-335) describes many of these methods and their applications. For example, Proffitt et. al. (*Cytometry* 24: 204-213 (1996)) describe
20 a semi-automated fluorescence digital imaging system for quantifying relative cell numbers in situ in a variety of tissue culture plate formats, especially 96-well microtiter plates. The system consists of an epifluorescence inverted microscope with a motorized stage, video camera, image intensifier, and a microcomputer with a PC-Vision digitizer. Turbo Pascal software controls the stage and scans the plate taking
25 multiple images per well. The software calculates total fluorescence per well, provides for daily calibration, and configures easily for a variety of tissue culture plate formats. Thresholding of digital images and reagents which fluoresce only when taken up by living cells are used to reduce background fluorescence without removing excess fluorescent reagent.

30 Scanning confocal microscope imaging (Go et al., (1997) *Analytical Biochemistry* 247:210-215; Goldman et al., (1995) *Experimental Cell Research* 221:311-319) and multiphoton microscope imaging (Denk et al., (1990) *Science*

248:73; Gratton et al., (1994) *Proc. of the Microscopical Society of America*, pp. 154-155) are also well established methods for acquiring high resolution images of microscopic samples. The principle advantage of these optical systems is the very shallow depth of focus, which allows features of limited axial extent to be resolved against the background. For example, it is possible to resolve internal cytoplasmic features of adherent cells from the features on the cell surface. Because scanning multiphoton imaging requires very short duration pulsed laser systems to achieve the high photon flux required, fluorescence lifetimes can also be measured in these systems (Lakowicz et al., (1992) *Anal. Biochem.* 202:316-330; Gerritsen et al. (1997), *J. of Fluorescence* 7:11-15)), providing additional capability for different detection modes. Small, reliable and relatively inexpensive laser systems, such as laser diode pumped lasers, are now available to allow multiphoton confocal microscopy to be applied in a fairly routine fashion.

A combination of the biological heterogeneity of cells in populations (Bright, et al., (1989). *J. Cell. Physiol.* 141:410; Giuliano, (1996) *Cell Motil. Cytoskel.* 35:237)) as well as the high spatial and temporal frequency of chemical and molecular information present within cells, makes it impossible to extract high-content information from populations of cells using existing whole microtiter plate readers. No existing high-content screening platform has been designed for multicolor, fluorescence-based screens using cells that are analyzed individually. Similarly, no method is currently available that combines automated fluid delivery to arrays of cells for the purpose of systematically screening compounds for the ability to induce a cellular response that is identified by HCS analysis, especially from cells grown in microtiter plates. Furthermore, no method exists in the art combining high throughput well-by-well measurements to identify "hits" in one assay followed by a second high content cell-by-cell measurement on the same plate of only those wells identified as hits.

The instant invention provides systems, methods, and screens that combine high throughput screening (HTS) and high content screening (HCS) that significantly improve target validation and candidate optimization by combining many cell screening formats with fluorescence-based molecular reagents and computer-based feature extraction, data analysis, and automation, resulting in increased quantity and speed of

data collection, shortened cycle times, and, ultimately, faster evaluation of promising drug candidates. The instant invention also provides for miniaturizing the methods, thereby allowing increased throughput, while decreasing the volumes of reagents and test compounds required in each assay.

5

SUMMARY OF THE INVENTION

In one aspect, the present invention relates to a method for analyzing cells comprising providing cells containing fluorescent reporter molecules in an array of locations, treating the cells in the array of locations with one or more reagents, 10 imaging numerous cells in each location with fluorescence optics, converting the optical information into digital data, utilizing the digital data to determine the distribution, environment or activity of the fluorescently labeled reporter molecules in the cells and the distribution of the cells, and interpreting that information in terms of a positive, negative or null effect of the compound being tested on the biological 15 function

In this embodiment, the method rapidly determines the distribution, environment, or activity of fluorescently labeled reporter molecules in cells for the purpose of screening large numbers of compounds for those that specifically affect particular biological functions. The array of locations may be a microtiter plate or a 20 microchip which is a microplate having cells in an array of locations. In a preferred embodiment, the method includes computerized means for acquiring, processing, displaying and storing the data received. In a preferred embodiment, the method further comprises automated fluid delivery to the arrays of cells. In another preferred embodiment, the information obtained from high throughput measurements on the 25 same plate are used to selectively perform high content screening on only a subset of the cell locations on the plate.

In another aspect of the present invention, a cell screening system is provided that comprises:

- a high magnification fluorescence optical system having a microscope 30 objective,

- an XY stage adapted for holding a plate containing an array of cells and having a means for moving the plate for proper alignment and focusing on the cell arrays;
- a digital camera;
- 5 • a light source having optical means for directing excitation light to cell arrays and a means for directing fluorescent light emitted from the cells to the digital camera; and
- 10 • a computer means for receiving and processing digital data from the digital camera wherein the computer means includes a digital frame grabber for receiving the images from the camera, a display for user interaction and display of assay results, digital storage media for data storage and archiving, and a means for control, acquisition, processing and display of results.

15 In a preferred embodiment, the cell screening system further comprises a computer screen operatively associated with the computer for displaying data. In another preferred embodiment, the computer means for receiving and processing digital data from the digital camera stores the data in a bioinformatics data base. In a further preferred embodiment, the cell screening system further comprises a reader that measures a signal from many or all the wells in parallel. In another preferred
20 embodiment, the cell screening system further comprises a mechanical-optical means for changing the magnification of the system, to allow changing modes between high throughput and high content screening. In another preferred embodiment, the cell screening system further comprises a chamber and control system to maintain the temperature, CO₂ concentration and humidity surrounding the plate at levels required to
25 keep cells alive. In a further preferred embodiment, the cell screening system utilizes a confocal scanning illumination and detection system.

30 In another aspect of the present invention, a machine readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute procedures for defining the distribution and activity of specific cellular constituents and processes is provided. In a preferred embodiment, the cell screening system comprises a high magnification fluorescence optical system with a stage adapted for holding cells and a means for moving the stage, a digital camera, a

light source for receiving and processing the digital data from the digital camera, and a computer means for receiving and processing the digital data from the digital camera. Preferred embodiments of the machine readable storage medium comprise programs consisting of a set of instructions for causing a cell screening system to execute the procedures set forth in Figures 9, 11, 12, 13, 14 or 15. Another preferred embodiment comprises a program consisting of a set of instructions for causing a cell screening system to execute procedures for detecting the distribution and activity of specific cellular constituents and processes. In most preferred embodiments, the cellular processes include, but are not limited to, nuclear translocation of a protein, cellular hypertrophy, apoptosis, and protease-induced translocation of a protein.

In another preferred embodiment, a variety of automated cell screening methods are provided, including screens to identify compounds that affect transcription factor activity, protein kinase activity, cell morphology, microtubule structure, apoptosis, receptor internalization, and protease-induced translocation of a protein.

In another aspect, the present invention provides recombinant nucleic acids encoding a protease biosensor, comprising:

- a. a first nucleic acid sequence that encodes at least one detectable polypeptide signal;
- b. a second nucleic acid sequence that encodes at least one protease recognition site, wherein the second nucleic acid sequence is operatively linked to the first nucleic acid sequence that encodes the at least one detectable polypeptide signal; and
- c. a third nucleic acid sequence that encodes at least one reactant target sequence, wherein the third nucleic acid sequence is operatively linked to the second nucleic acid sequence that encodes the at least one protease recognition site.

The present invention also provides the recombinant expression vectors capable of expressing the recombinant nucleic acids encoding protease biosensors, as well as genetically modified host cells that are transfected with the expression vectors.

The invention further provides recombinant protease biosensors, comprising

- a. a first domain comprising at least one detectable polypeptide signal;
- b. a second domain comprising at least one protease recognition site; and
- c. a third domain comprising at least one reactant target sequence;

wherein the first domain and the third domain are separated by the second domain.

In a further aspect, the present invention involves assays and reagents for characterizing a sample for the presence of a toxin. The method comprises the use of
5 detector, classifier, and identifier classes of toxin biosensors to provide for various levels of toxin characterization.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a diagram of the components of the cell-based scanning system.

10 **Figure 2** shows a schematic of the microscope subassembly.

Figure 3 shows the camera subassembly.

Figure 4 illustrates cell scanning system process.

Figure 5 illustrates a user interface showing major functions to guide the user.

15 **Figure 6** is a block diagram of the two platform architecture of the Dual Mode System for Cell Based Screening in which one platform uses a telescope lens to read all wells of a microtiter plate and a second platform that uses a higher magnification lens to read individual cells in a well.

20 **Figure 7** is a detail of an optical system for a single platform architecture of the Dual Mode System for Cell Based Screening that uses a moveable 'telescope' lens to read all wells of a microtiter plate and a moveable higher magnification lens to read individual cells in a well.

Figure 8 is an illustration of the fluid delivery system for acquiring kinetic data on the Cell Based Screening System.

Figure 9 is a flow chart of processing step for the cell-based scanning system.

25 **Figure 10 A-J** illustrates the strategy of the Nuclear Translocation Assay.

Figure 11 is a flow chart defining the processing steps in the Dual Mode System for Cell Based Screening combining high throughput and high content screening of microtiter plates.

30 **Figure 12** is a flow chart defining the processing steps in the High Throughput mode of the System for Cell Based Screening.

Figure 13 is a flow chart defining the processing steps in the High Content mode of the System for Cell Based Screening.

Figure 14 is a flow chart defining the processing steps required for acquiring kinetic data in the High Content mode of the System for Cell Based Screening.

Figure 15 is a flow chart defining the processing steps performed within a well during the acquisition of kinetic data.

5 Figure 16 is an example of data from a known inhibitor of translocation.

Figure 17 is an example of data from a known stimulator of translocation.

Figure 18 illustrates data presentation on a graphical display.

Figure 19 is an illustration of the data from the High Throughput mode of the System for Cell Based Screening, an example of the data passed to the High Content mode, the data acquired in the high content mode, and the results of the analysis of that data,

10 Figure 20 shows the measurement of a drug-induced cytoplasm to nuclear translocation.

Figure 21 illustrates a graphical user interface of the measurement shown in Figure 20.

15 Figure 22 illustrates a graphical user interface, with data presentation, of the measurement shown in Fig. 20.

Figure 23 is a graph representing the kinetic data obtained from the measurements depicted in Fig. 20.

Figure 24 details a high-content screen of drug-induced apoptosis.

20 Figure 25. Graphs depicting changes in morphology upon induction of apoptosis. Staurosporine (A) and paclitaxel (B) induce classic nuclear fragmentation in L929 cells. BHK cells exhibit concentration dependent changes in response to staurosporine (C), but a more classical response to paclitaxel (D). MCF-7 cells exhibit either nuclear condensation (E) or fragmentation (F) in response to staurosporine and paclitaxel, respectively. In all cases, cells were exposed to the compounds for 30 hours.

25 Figure 26 illustrates the dose response of cells to staurosporine in terms of both nuclear size and nuclear perimeter convolution.

30 Figure 27. Graphs depicting induction of apoptosis by staurosporine and paclitaxel leading to changes in peri-nuclear f-actin content. (A, B) Both apoptotic stimulators induce dose-dependent increases in f-actin content in L929 cells. (C) In BHK cells, staurosporine induces a dose-dependent increase in f-actin, whereas paclitaxel (D) produces results that are more variable. (E) MCF-7 cells exhibit either a decrease or increase depending on the concentration of staurosporine. (F) Paclitaxel induced

changes in f-actin content were highly variable and not significant. Cells were exposed to the compounds for 30 hours.

Figure 28. Graphs depicting mitochondrial changes in response to induction of apoptosis. L929 (A,B) and BHK (C,D) cells responded to both staurosporine (A,C) and paclitaxel (B,D) with increases in mitochondrial mass. MCF-7 cells exhibit either a decrease in membrane potential (E, staurosporine) or an increase in mitochondrial mass (F, paclitaxel) depending on the stimulus. Cells were exposed to the compounds for 30 hours. 28G is a graph showing the simultaneous measurement of staurosporine effects on mitochondrial mass and mitochondrial potential in BHK cells.

Figure 29 shows the nucleic acid and amino acid sequence for various types of protease biosensor domains. (A) Signal sequences. (B) Protease recognition sites. (C) Product/Reactant target sequences

Figure 30 shows schematically shows some basic organization of domains in the protease biosensors of the invention.

Figure 31 is a schematic diagram of a specific 3-domain protease biosensor.

Figure 32 is a photograph showing the effect of stimulation of apoptosis by cis-platin on BHK cells transfected with an expression vector that expresses the caspase biosensor shown in Figure 32.

Figure 33 is a schematic diagram of a specific 4-domain protease biosensor.

Figure 34 is a schematic diagram of a specific 4-domain protease biosensor, containing a nucleolar localization signal.

Figure 35 is a schematic diagram of a specific 5-domain protease biosensor.

Figure 36 shows the differential response in a dual labeling assay of the p38 MAPK and NF- κ B pathways across three model toxins and two different cell types.

Treatments marked with an asterisk are different from controls at a 99% confidence level ($p < 0.01$).

DETAILED DESCRIPTION OF THE INVENTION

All cited patents, patent applications and other references are hereby incorporated by reference in their entirety.

As used herein, the following terms have the specified meaning:

Markers of cellular domains. Luminescent probes that have high affinity for specific cellular constituents including specific organelles or molecules. These probes can either be small luminescent molecules or fluorescently tagged macromolecules used as "labeling reagents", "environmental indicators", or "biosensors."

5 *Labeling reagents.* Labeling reagents include, but are not limited to, luminescently labeled macromolecules including fluorescent protein analogs and biosensors, luminescent macromolecular chimeras including those formed with the green fluorescent protein and mutants thereof, luminescently labeled primary or secondary antibodies that react with cellular antigens involved in a physiological
10 response, luminescent stains, dyes, and other small molecules.

Markers of cellular translocations. Luminescently tagged macromolecules or organelles that move from one cell domain to another during some cellular process or physiological response. Translocation markers can either simply report location relative to the markers of cellular domains or they can also be "biosensors" that report
15 some biochemical or molecular activity as well.

Biosensors. Macromolecules consisting of a biological functional domain and a luminescent probe or probes that report the environmental changes that occur either internally or on their surface. A class of luminescently labeled macromolecules designed to sense and report these changes have been termed "fluorescent-protein
20 biosensors". The protein component of the biosensor provides a highly evolved molecular recognition moiety. A fluorescent molecule attached to the protein component in the proximity of an active site transduces environmental changes into fluorescence signals that are detected using a system with an appropriate temporal and spatial resolution such as the cell scanning system of the present invention. Because
25 the modulation of native protein activity within the living cell is reversible, and because fluorescent-protein biosensors can be designed to sense reversible changes in protein activity, these biosensors are essentially reusable.

Disease associated sequences ("DAS"). This term refers to nucleic acid sequences identified by standard techniques, such as primary DNA sequence data,
30 genomic methods such as subtraction hybridization and RADE, and proteomic methods in combination with reverse genetics, as being of drug candidate compounds. The term does not mean that the sequence is only associated with a disease state.

High content screening (HCS) can be used to measure the effects of drugs on complex molecular events such as signal transduction pathways, as well as cell functions including, but not limited to, apoptosis, cell division, cell adhesion, locomotion, exocytosis, and cell-cell communication. Multicolor fluorescence permits multiple targets and cell processes to be assayed in a single screen. Cross-correlation of cellular responses will yield a wealth of information required for target validation and lead optimization.

In one aspect of the present invention, a cell screening system is provided comprising a high magnification fluorescence optical system having a microscope objective, an XY stage adapted for holding a plate with an array of locations for holding cells and having a means for moving the plate to align the locations with the microscope objective and a means for moving the plate in the direction to effect focusing; a digital camera; a light source having optical means for directing excitation light to cells in the array of locations and a means for directing fluorescent light emitted from the cells to the digital camera; and a computer means for receiving and processing digital data from the digital camera wherein the computer means includes: a digital frame grabber for receiving the images from the camera, a display for user interaction and display of assay results, digital storage media for data storage and archiving, and means for control, acquisition, processing and display of results.

Figure 1 is a schematic diagram of a preferred embodiment of the cell scanning system. An inverted fluorescence microscope is used 1, such as a Zeiss Axiovert inverted fluorescence microscope which uses standard objectives with magnification of 1-100x to the camera, and a white light source (e.g. 100W mercury-arc lamp or 75W xenon lamp) with power supply 2. There is an XY stage 3 to move the plate 4 in the XY direction over the microscope objective. A Z-axis focus drive 5 moves the objective in the Z direction for focusing. A joystick 6 provides for manual movement of the stage in the XYZ direction. A high resolution digital camera 7 acquires images from each well or location on the plate. There is a camera power supply 8, an automation controller 9 and a central processing unit 10. The PC 11 provides a display 12 and has associated software. The printer 13 provides for printing of a hard copy record.

Figure 2 is a schematic of one embodiment of the microscope assembly 1 of the invention, showing in more detail the XY stage 3, Z-axis focus drive 5, joystick 6, light source 2, and automation controller 9. Cables to the computer 15 and microscope 16, respectively, are provided. In addition, Figure 2 shows a 96 well microtiter plate 17 which is moved on the XY stage 3 in the XY direction. Light from the light source 2 passes through the PC controlled shutter 18 to a motorized filter wheel 19 with excitation filters 20. The light passes into filter cube 25 which has a dichroic mirror 26 and an emission filter 27. Excitation light reflects off the dichroic mirror to the wells in the microtiter plate 17 and fluorescent light 28 passes through the dichroic mirror 26 and the emission filter 27 and to the digital camera 7.

Figure 3 shows a schematic drawing of a preferred camera assembly. The digital camera 7, which contains an automatic shutter for exposure control and a power supply 31, receives fluorescent light 28 from the microscope assembly. A digital cable 30 transports digital signals to the computer.

The standard optical configurations described above use microscope optics to directly produce an enlarged image of the specimen on the camera sensor in order to capture a high resolution image of the specimen. This optical system is commonly referred to as 'wide field' microscopy. Those skilled in the art of microscopy will recognize that a high resolution image of the specimen can be created by a variety of other optical systems, including, but not limited to, standard scanning confocal detection of a focused point or line of illumination scanned over the specimen (Go et al. 1997, *supra*), and multi-photon scanning confocal microscopy (Denk et al., 1990, *supra*), both of which can form images on a CCD detector or by synchronous digitization of the analog output of a photomultiplier tube.

In screening applications, it is often necessary to use a particular cell line, or primary cell culture, to take advantage of particular features of those cells. Those skilled in the art of cell culture will recognize that some cell lines are contact inhibited, meaning that they will stop growing when they become surrounded by other cells, while other cell lines will continue to grow under those conditions and the cells will literally pile up, forming many layers. An example of such a cell line is the HEK 293 (ATCC CRL-1573) line. An optical system that can acquire images of single cell layers in multilayer preparations is required for use with cell lines that tend to form

layers. The large depth of field of wide field microscopes produces an image that is a projection through the many layers of cells, making analysis of subcellular spatial distributions extremely difficult in layer-forming cells. Alternatively, the very shallow depth of field that can be achieved on a confocal microscope, (about one micron),
5 allows discrimination of a single cell layer at high resolution, simplifying the determination of the subcellular spatial distribution. Similarly, confocal imaging is preferable when detection modes such as fluorescence lifetime imaging are required.

The output of a standard confocal imaging attachment for a microscope is a digital image that can be converted to the same format as the images produced by the
10 other cell screening system embodiments described above, and can therefore be processed in exactly the same way as those images. The overall control, acquisition and analysis in this embodiment is essentially the same. The optical configuration of the confocal microscope system, is essentially the same as that described above, except for the illuminator and detectors. Illumination and detection systems required for
15 confocal microscopy have been designed as accessories to be attached to standard microscope optical systems such as that of the present invention (Zeiss, Germany). These alternative optical systems therefore can be easily integrated into the system as described above.

Figure 4 illustrates an alternative embodiment of the invention in which cell
20 arrays are in microwells 40 on a microplate 41, described in co-pending U.S. Application S/N 08/865,341, incorporated by reference herein in its entirety. Typically the microplate is 20 mm by 30 mm as compared to a standard 96 well microtiter plate which is 86 mm by 129 mm. The higher density array of cells on a microplate allows the microplate to be imaged at a low resolution of a few microns per pixel for high
25 throughput and particular locations on the microplate to be imaged at a higher resolution of less than 0.5 microns per pixel. These two resolution modes help to improve the overall throughput of the system.

The microplate chamber 42 serves as a microfluidic delivery system for the addition of compounds to cells. The microplate 41 in the microplate chamber 42 is
30 placed in an XY microplate reader 43. Digital data is processed as described above. The small size of this microplate system increases throughput, minimizes reagent volume and allows control of the distribution and placement of cells for fast and precise

cell-based analysis. Processed data can be displayed on a PC screen 11 and made part of a bioinformatics data base 44. This data base not only permits storage and retrieval of data obtained through the methods of this invention, but also permits acquisition and storage of external data relating to cells. Figure 5 is a PC display which illustrates the operation of the software.

In an alternative embodiment, a high throughput system (HTS) is directly coupled with the HCS either on the same platform or on two separate platforms connected electronically (e.g. via a local area network). This embodiment of the invention, referred to as a dual mode optical system, has the advantage of increasing the throughput of a HCS by coupling it with a HTS and thereby requiring slower high resolution data acquisition and analysis only on the small subset of wells that show a response in the coupled HTS.

High throughput 'whole plate' reader systems are well known in the art and are commonly used as a component of an HTS system used to screen large numbers of compounds (Beggs (1997), *J. of Biomolec. Screening* 2:71-78; Macaffrey et al., (1996) *J. Biomolec. Screening* 1:187-190).

In one embodiment of dual mode cell based screening, a two platform architecture in which high throughput acquisition occurs on one platform and high content acquisition occurs on a second platform is provided (Figure 6). Processing occurs on each platform independently, with results passed over a network interface, or a single controller is used to process the data from both platforms.

As illustrated in Figure 6, an exemplified two platform dual mode optical system consists of two light optical instruments, a high throughput platform 60 and a high content platform 65, which read fluorescent signals emitted from cells cultured in microtiter plates or microwell arrays on a microplate, and communicate with each other via an electronic connection 64. The high throughput platform 60 analyzes all the wells in the whole plate either in parallel or rapid serial fashion. Those skilled in the art of screening will recognize that there are a many such commercially available high throughput reader systems that could be integrated into a dual mode cell based screening system (Topcount (Packard Instruments, Meriden, CT); Spectramax, Lumiskan (Molecular Devices, Sunnyvale, CA); Fluoroscan (Labsystems, Beverly, MA)). The high content platform 65, as described above, scans from well to well and

acquires and analyzes high resolution image data collected from individual cells within a well.

The HTS software, residing on the system's computer 62, controls the high throughput instrument, and results are displayed on the monitor 61. The HCS software, residing on it's computer system 67, controls the high content instrument hardware 65, optional devices (e.g. plate loader, environmental chamber, fluid dispenser), analyzes digital image data from the plate, displays results on the monitor 66 and manages data measured in an integrated database. The two systems can also share a single computer, in which case all data would be collected, processed and displayed on that computer, without the need for a local area network to transfer the data. Microtiter plates are transferred from the high throughput system to the high content system 63 either manually or by a robotic plate transfer device, as is well known in the art (Beggs (1997), *supra*; McAffrey (1996), *supra*).

In a preferred embodiment, the dual mode optical system utilizes a single platform system (Figure 7). It consists of two separate optical modules, an HCS module 203 and an HTS module 209 that can be independently or collectively moved so that only one at a time is used to collect data from the microtiter plate 201. The microtiter plate 201 is mounted in a motorized X,Y stage so it can be positioned for imaging in either HTS or HCS mode. After collecting and analyzing the HTS image data as described below, the HTS optical module 209 is moved out of the optical path and the HCS optical module 203 is moved into place.

The optical module for HTS 209 consists of a projection lens 214, excitation wavelength filter 213 and dichroic mirror 210 which are used to illuminate the whole bottom of the plate with a specific wavelength band from a conventional microscope lamp system (not illustrated). The fluorescence emission is collected through the dichroic mirror 210 and emission wavelength filter 211 by a lens 212 which forms an image on the camera 216 with sensor 215.

The optical module for HCS 203 consists of a projection lens 208, excitation wavelength filter 207 and dichroic mirror 204 which are used to illuminate the back aperture of the microscope objective 202, and thereby the field of that objective, from a standard microscope illumination system (not shown). The fluorescence emission is

collected by the microscope objective 202, passes through the dichroic mirror 204 and emission wavelength filter 205 and is focused by a tube lens 206 which forms an image on the same camera 216 with sensor 215.

In an alternative embodiment of the present invention, the cell screening system further comprises a fluid delivery device for use with the live cell embodiment of the method of cell screening (see below). Figure 8 exemplifies a fluid delivery device for use with the system of the invention. It consists of a bank of 12 syringe pumps 701 driven by a single motor drive. Each syringe 702 is sized according to the volume to be delivered to each well, typically between 1 and 100 μ L. Each syringe is attached via flexible tubing 703 to a similar bank of connectors which accept standard pipette tips 705. The bank of pipette tips are attached to a drive system so they can be lowered and raised relative to the microtiter plate 706 to deliver fluid to each well. The plate is mounted on an X,Y stage, allowing movement relative to the optical system 707 for data collection purposes. This set-up allows one set of pipette tips, or even a single pipette tip, to deliver reagent to all the wells on the plate. The bank of syringe pumps can be used to deliver fluid to 12 wells simultaneously, or to fewer wells by removing some of the tips.

In another aspect, the present invention provides a method for analyzing cells comprising providing an array of locations which contain multiple cells wherein the cells contain one or more fluorescent reporter molecules; scanning multiple cells in each of the locations containing cells to obtain fluorescent signals from the fluorescent reporter molecule in the cells; converting the fluorescent signals into digital data; and utilizing the digital data to determine the distribution, environment or activity of the fluorescent reporter molecule within the cells.

Cell Arrays

Screening large numbers of compounds for activity with respect to a particular biological function requires preparing arrays of cells for parallel handling of cells and reagents. Standard 96 well microtiter plates which are 86 mm by 129 mm, with 6mm diameter wells on a 9mm pitch, are used for compatibility with current automated loading and robotic handling systems. The microplate is typically 20 mm by 30 mm, with cell locations that are 100-200 microns in dimension on a pitch of about 500

microns. Methods for making microplates are described in U.S. Patent Application Serial No. 08/865,341, incorporated by reference herein in its entirety. Microplates may consist of coplanar layers of materials to which cells adhere, patterned with materials to which cells will not adhere, or etched 3-dimensional surfaces of similarly patterned materials. For the purpose of the following discussion, the terms 'well' and 'microwell' refer to a location in an array of any construction to which cells adhere and within which the cells are imaged. Microplates may also include fluid delivery channels in the spaces between the wells. The smaller format of a microplate increases the overall efficiency of the system by minimizing the quantities of the reagents, storage and handling during preparation and the overall movement required for the scanning operation. In addition, the whole area of the microplate can be imaged more efficiently, allowing a second mode of operation for the microplate reader as described later in this document.

Fluorescence Reporter Molecules

A major component of the new drug discovery paradigm is a continually growing family of fluorescent and luminescent reagents that are used to measure the temporal and spatial distribution, content, and activity of intracellular ions, metabolites, macromolecules, and organelles. Classes of these reagents include labeling reagents that measure the distribution and amount of molecules in living and fixed cells, environmental indicators to report signal transduction events in time and space, and fluorescent protein biosensors to measure target molecular activities within living cells. A multiparameter approach that combines several reagents in a single cell is a powerful new tool for drug discovery.

The method of the present invention is based on the high affinity of fluorescent or luminescent molecules for specific cellular components. The affinity for specific components is governed by physical forces such as ionic interactions, covalent bonding (which includes chimeric fusion with protein-based chromophores, fluorophores, and lumiphores), as well as hydrophobic interactions, electrical potential, and, in some cases, simple entrapment within a cellular component. The luminescent probes can be small molecules, labeled macromolecules, or genetically engineered proteins, including, but not limited to green fluorescent protein chimeras.

Those skilled in this art will recognize a wide variety of fluorescent reporter molecules that can be used in the present invention, including, but not limited to, fluorescently labeled biomolecules such as proteins, phospholipids and DNA hybridizing probes. Similarly, fluorescent reagents specifically synthesized with particular chemical properties of binding or association have been used as fluorescent reporter molecules (Barak et al., (1997), *J. Biol. Chem.* 272:27497-27500; Southwick et al., (1990), *Cytometry* 11:418-430; Tsien (1989) in *Methods in Cell Biology*, Vol. 29 Taylor and Wang (eds.), pp. 127-156). Fluorescently labeled antibodies are particularly useful reporter molecules due to their high degree of specificity for attaching to a single molecular target in a mixture of molecules as complex as a cell or tissue.

The luminescent probes can be synthesized within the living cell or can be transported into the cell via several non-mechanical modes including diffusion, facilitated or active transport, signal-sequence-mediated transport, and endocytotic or pinocytotic uptake. Mechanical bulk loading methods, which are well known in the art, can also be used to load luminescent probes into living cells (Barber et al. (1996), *Neuroscience Letters* 207:17-20; Bright et al. (1996), *Cytometry* 24:226-233; McNeil (1989) in *Methods in Cell Biology*, Vol. 29, Taylor and Wang (eds.), pp. 153-173). These methods include electroporation and other mechanical methods such as scrape-loading, bead-loading, impact-loading, syringe-loading, hypertonic and hypotonic loading. Additionally, cells can be genetically engineered to express reporter molecules, such as GFP, coupled to a protein of interest as previously described (Chalfie and Prasher U.S. Patent No. 5,491,084; Cubitt et al. (1995), *Trends in Biochemical Science* 20:448-455).

Once in the cell, the luminescent probes accumulate at their target domain as a result of specific and high affinity interactions with the target domain or other modes of molecular targeting such as signal-sequence-mediated transport. Fluorescently labeled reporter molecules are useful for determining the location, amount and chemical environment of the reporter. For example, whether the reporter is in a lipophilic membrane environment or in a more aqueous environment can be determined (Giuliano et al. (1995), *Ann. Rev. of Biophysics and Biomolecular Structure* 24:405-434; Giuliano and Taylor (1995), *Methods in Neuroscience* 27:1-16). The pH environment of the reporter can be determined (Bright et al. (1989), *J. Cell Biology* 104:1019-1033;

Giuliano et al. (1987), *Anal. Biochem.* 167:362-371; Thomas et al. (1979), *Biochemistry* 18:2210-2218). It can be determined whether a reporter having a chelating group is bound to an ion, such as Ca^{++} , or not (Bright et al. (1989), In *Methods in Cell Biology*, Vol. 30, Taylor and Wang (eds.), pp. 157-192; Shimoura et al. (1988), *J. of Biochemistry* (Tokyo) 251:405-410; Tsien (1989) In *Methods in Cell Biology*, Vol. 30, Taylor and Wang (eds.), pp. 127-156).

Furthermore, certain cell types within an organism may contain components that can be specifically labeled that may not occur in other cell types. For example, epithelial cells often contain polarized membrane components. That is, these cells asymmetrically distribute macromolecules along their plasma membrane. Connective or supporting tissue cells often contain granules in which are trapped molecules specific to that cell type (e.g., heparin, histamine, serotonin, etc.). Most muscular tissue cells contain a sarcoplasmic reticulum, a specialized organelle whose function is to regulate the concentration of calcium ions within the cell cytoplasm. Many nervous tissue cells contain secretory granules and vesicles in which are trapped neurohormones or neurotransmitters. Therefore, fluorescent molecules can be designed to label not only specific components within specific cells, but also specific cells within a population of mixed cell types.

Those skilled in the art will recognize a wide variety of ways to measure fluorescence. For example, some fluorescent reporter molecules exhibit a change in excitation or emission spectra, some exhibit resonance energy transfer where one fluorescent reporter loses fluorescence, while a second gains in fluorescence, some exhibit a loss (quenching) or appearance of fluorescence, while some report rotational movements (Giuliano et al. (1995), *Ann. Rev. of Biophysics and Biomol. Structure* 24:405-434; Giuliano et al. (1995), *Methods in Neuroscience* 27:1-16).

Scanning cell arrays

Referring to Figure 9, a preferred embodiment is provided to analyze cells that comprises operator-directed parameters being selected based on the assay being conducted, data acquisition by the cell screening system on the distribution of fluorescent signals within a sample, and interactive data review and analysis. At the start of an automated scan the operator enters information 100 that describes the sample, specifies the filter settings and fluorescent channels to match the biological

labels being used and the information sought, and then adjusts the camera settings to match the sample brightness. For flexibility to handle a range of samples, the software allows selection of various parameter settings used to identify nuclei and cytoplasm, and selection of different fluorescent reagents, identification of cells of interest based on morphology or brightness, and cell numbers to be analyzed per well. These parameters are stored in the system's for easy retrieval for each automated run. The system's interactive cell identification mode simplifies the selection of morphological parameter limits such as the range of size, shape, and intensity of cells to be analyzed. The user specifies which wells of the plate the system will scan and how many fields or how many cells to analyze in each well. Depending on the setup mode selected by the user at step 101, the system either automatically pre-focuses the region of the plate to be scanned using an autofocus procedure to "find focus" of the plate 102 or the user interactively pre-focuses 103 the scanning region by selecting three "tag" points which define the rectangular area to be scanned. A least-squares fit "focal plane model" is then calculated from these tag points to estimate the focus of each well during an automated scan. The focus of each well is estimated by interpolating from the focal plane model during a scan.

During an automated scan, the software dynamically displays the scan status, including the number of cells analyzed, the current well being analyzed, images of each independent wavelength as they are acquired, and the result of the screen for each well as it is determined. The plate 4 (Figure 1) is scanned in a serpentine style as the software automatically moves the motorized microscope XY stage 3 from well to well and field to field within each well of a 96-well plate. Those skilled in the programming art will recognize how to adapt software for scanning of other microplate formats such as 24, 48, and 384 well plates. The scan pattern of the entire plate as well as the scan pattern of fields within each well are programmed. The system adjusts sample focus with an autofocus procedure 104 (Figure 9) through the Z axis focus drive 5, controls filter selection via a motorized filter wheel 19, and acquires and analyzes images of up to four different colors ("channels" or "wavelengths").

The autofocus procedure is called at a user selected frequency, typically for the first field in each well and then once every 4 to 5 fields within each well. The autofocus procedure calculates the starting Z-axis point by interpolating from the pre-calculated

plane focal model. Starting a programmable distance above or below this set point, the procedure moves the mechanical Z-axis through a number of different positions, acquires an image at each position, and finds the maximum of a calculated focus score that estimates the contrast of each image. The Z position of the image with the maximum focus score determines the best focus for a particular field. Those skilled in the art will recognize this as a variant of automatic focusing methods as described in Harms et al. in *Cytometry* 5 (1984), 236-243, Groen et al. in *Cytometry* 6 (1985), 81-91, and Firestone et al. in *Cytometry* 12 (1991), 195-206.

For image acquisition, the camera's exposure time is separately adjusted for each dye to ensure a high-quality image from each channel. Software procedures can be called, at the user's option, to correct for registration shifts between wavelengths by accounting for linear (X and Y) shifts between wavelengths before making any further measurements. The electronic shutter 18 is controlled so that sample photo-bleaching is kept to a minimum. Background shading and uneven illumination can be corrected by the software using methods known in the art (Bright et al. (1987), *J. Cell Biol.* 104:1019-1033).

In one channel, images are acquired of a primary marker 105 (Figure 9) (typically cell nuclei counterstained with DAPI or PI fluorescent dyes) which are segmented ("identified") using an adaptive thresholding procedure. The adaptive thresholding procedure 106 is used to dynamically select the threshold of an image for separating cells from the background. The staining of cells with fluorescent dyes can vary to an unknown degree across cells in a microtiter plate sample as well as within images of a field of cells within each well of a microtiter plate. This variation can occur as a result of sample preparation and/or the dynamic nature of cells. A global threshold is calculated for the complete image to separate the cells from background and account for field to field variation. These global adaptive techniques are variants of those described in the art. (Kittler et al. in *Computer Vision, Graphics, and Image Processing* 30 (1985), 125-147, Ridler et al. in *IEEE Trans. Systems, Man, and Cybernetics* (1978), 630-632.)

An alternative adaptive thresholding method utilizes local region thresholding in contrast to global image thresholding. Image analysis of local regions leads to better overall segmentation since staining of cell nuclei (as well as other labeled components)

can vary across an image. Using this global/local procedure, a reduced resolution image (reduced in size by a factor of 2 to 4) is first globally segmented (using adaptive thresholding) to find regions of interest in the image. These regions then serve as guides to more fully analyze the same regions at full resolution. A more localized threshold is then calculated (again using adaptive thresholding) for each region of interest.

The output of the segmentation procedure is a binary image wherein the objects are white and the background is black. This binary image, also called a mask in the art, is used to determine if the field contains objects 107. The mask is labeled with a blob labeling method whereby each object (or blob) has a unique number assigned to it. Morphological features, such as area and shape, of the blobs are used to differentiate blobs likely to be cells from those that are considered artifacts. The user pre-sets the morphological selection criteria by either typing in known cell morphological features or by using the interactive training utility. If objects of interest are found in the field, images are acquired for all other active channels 108, otherwise the stage is advanced to the next field 109 in the current well. Each object of interest is located in the image for further analysis 110. The software determines if the object meets the criteria for a valid cell nucleus 111 by measuring its morphological features (size and shape). For each valid cell, the XYZ stage location is recorded, a small image of the cell is stored, and features are measured 112.

The cell scanning method of the present invention can be used to perform many different assays on cellular samples by applying a number of analytical methods simultaneously to measure features at multiple wavelengths. An example of one such assay provides for the following measurements:

1. The total fluorescent intensity within the cell nucleus for colors 1-4
2. The area of the cell nucleus for color 1 (the primary marker)
3. The shape of the cell nucleus for color 1 is described by three shape features:
 - a) perimeter squared area
 - b) box area ratio
 - c) height width ratio
4. The average fluorescent intensity within the cell nucleus for colors 1-4 (i.e. #1 divided by #2)
5. The total fluorescent intensity of a ring outside the nucleus (see Figure 10) that represents fluorescence of the cell's cytoplasm (cytoplasmic mask) for colors 2-4

6. The area of the cytoplasmic mask
7. The average fluorescent intensity of the cytoplasmic mask for colors 2-4 (i.e. #5 divided by #6)
8. The ratio of the average fluorescent intensity of the cytoplasmic mask to average fluorescent intensity within the cell nucleus for colors 2-4 (i.e. #7 divided by #4)
9. The difference of the average fluorescent intensity of the cytoplasmic mask and the average fluorescent intensity within the cell nucleus for colors 2-4 (i.e. #7 minus #4)
10. The number of fluorescent domains (also call spots, dots, or grains) within the cell nucleus for colors 2-4

Features 1 through 4 are general features of the different cell screening assays of the invention. These steps are commonly used in a variety of image analysis applications and are well known in art (Russ (1992) *The Image Processing Handbook*, CRC Press Inc.; Gonzales et al. (1987), *Digital Image Processing*. Addison-Wesley Publishing Co. pp. 391-448). Features 5-9 have been developed specifically to provide measurements of a cell's fluorescent molecules within the local cytoplasmic region of the cell and the translocation (i.e. movement) of fluorescent molecules from the cytoplasm to the nucleus. These features (steps 5-9) are used for analyzing cells in microplates for the inhibition of nuclear translocation. For example, inhibition of nuclear translocation of transcription factors provides a novel approach to screening intact cells (detailed examples of other types of screens will be provided below). A specific method measures the amount of probe in the nuclear region (feature 4) versus the local cytoplasmic region (feature 7) of each cell. Quantification of the difference between these two sub-cellular compartments provides a measure of cytoplasm-nuclear translocation (feature 9).

Feature 10 describes a screen used for counting of DNA or RNA probes within the nuclear region in colors 2-4. For example, probes are commercially available for identifying chromosome-specific DNA sequences (Life Technologies, Gaithersburg, MD; Genosys, Woodlands, TX; Biotechnologies, Inc., Richmond, CA; Bio 101, Inc., Vista, CA) Cells are three-dimensional in nature and when examined at a high magnification under a microscope one probe may be in-focus while another may be completely out-of-focus. The cell screening method of the present invention provides for detecting three-dimensional probes in nuclei by acquiring images from multiple focal planes. The software moves the Z-axis motor drive 5 (Figure 1) in small steps

where the step distance is user selected to account for a wide range of different nuclear diameters. At each of the focal steps, an image is acquired. The maximum gray-level intensity from each pixel in each image is found and stored in a resulting maximum projection image. The maximum projection image is then used to count the probes. The
5 above method works well in counting probes that are not stacked directly above or below another one. To account for probes stacked on top of each other in the Z-direction, users can select an option to analyze probes in each of the focal planes acquired. In this mode, the scanning system performs the maximum plane projection method as discussed above, detects probe regions of interest in this image, then further
10 analyzes these regions in all the focal plane images.

After measuring cell features 112 (Figure 9), the system checks if there are any unprocessed objects in the current field 113. If there are any unprocessed objects, it locates the next object 110 and determines whether it meets the criteria for a valid cell nucleus 111, and measures its features. Once all the objects in the current field are
15 processed, the system determines whether analysis of the current plate is complete 114; if not, it determines the need to find more cells in the current well 115. If the need exists, the system advances the XYZ stage to the next field within the current well 109 or advances the stage to the next well 116 of the plate.

After a plate scan is complete, images and data can be reviewed with the
20 system's image review, data review, and summary review facilities. All images, data, and settings from a scan are archived in the system's database for later review or for interfacing with a network information management system. Data can also be exported to other third-party statistical packages to tabulate results and generate other reports. Users can review the images alone of every cell analyzed by the system with an
25 interactive image review procedure 117. The user can review data on a cell-by-cell basis using a combination of interactive graphs, a data spreadsheet of measured features, and images of all the fluorescence channels of a cell of interest with the interactive cell-by-cell data review procedure 118. Graphical plotting capabilities are provided in which data can be analyzed via interactive graphs such as histograms and
30 scatter plots. Users can review summary data that are accumulated and summarized for all cells within each well of a plate with an interactive well-by-well data review

procedure 119. Hard copies of graphs and images can be printed on a wide range of standard printers.

As a final phase of a complete scan, reports can be generated on one or more statistics of the measured features. Users can generate a graphical report of data summarized on a well-by-well basis for the scanned region of the plate using an interactive report generation procedure 120. This report includes a summary of the statistics by well in tabular and graphical format and identification information on the sample. The report window allows the operator to enter comments about the scan for later retrieval. Multiple reports can be generated on many statistics and be printed with the touch of one button. Reports can be previewed for placement and data before being printed.

The above-recited embodiment of the method operates in a single high resolution mode referred to as the high content screening (HCS) mode. The HCS mode provides sufficient spatial resolution within a well (on the order of 1 μm) to define the distribution of material within the well, as well as within individual cells in the well. The high degree of information content accessible in that mode, comes at the expense of speed and complexity of the required signal processing.

In an alternative embodiment, a high throughput system (HTS) is directly coupled with the HCS either on the same platform or on two separate platforms connected electronically (e.g. via a local area network). This embodiment of the invention, referred to as a dual mode optical system, has the advantage of increasing the throughput of an HCS by coupling it with an HTS and thereby requiring slower high resolution data acquisition and analysis only on the small subset of wells that show a response in the coupled HTS.

High throughput 'whole plate' reader systems are well known in the art and are commonly used as a component of an HTS system used to screen large numbers of compounds (Beggs et al. (1997), *supra*; McCaffrey et al. (1996), *supra*). The HTS of the present invention is carried out on the microtiter plate or microwell array by reading many or all wells in the plate simultaneously with sufficient resolution to make determinations on a well-by-well basis. That is, calculations are made by averaging the total signal output of many or all the cells or the bulk of the material in each well.

Wells that exhibit some defined response in the HTS (the 'hits') are flagged by the system. Then on the same microtiter plate or microwell array, each well identified as a hit is measured via HCS as described above. Thus, the dual mode process involves:

1. Rapidly measuring numerous wells of a microtiter plate or microwell array,
- 5 2. Interpreting the data to determine the overall activity of fluorescently labeled reporter molecules in the cells on a well-by-well basis to identify "hits" (wells that exhibit a defined response),
3. Imaging numerous cells in each "hit" well, and
- 10 4. Interpreting the digital image data to determine the distribution, environment or activity of the fluorescently labeled reporter molecules in the individual cells (i.e. intracellular measurements) and the distribution of the cells to test for specific biological functions

In a preferred embodiment of dual mode processing (Figure 11), at the start of a run 301, the operator enters information 302 that describes the plate and its contents, specifies the filter settings and fluorescent channels to match the biological labels being used, the information sought and the camera settings to match the sample brightness. These parameters are stored in the system's database for easy retrieval for each automated run. The microtiter plate or microwell array is loaded into the cell screening system 303 either manually or automatically by controlling a robotic loading device. An optional environmental chamber 304 is controlled by the system to maintain the temperature, humidity and CO₂ levels in the air surrounding live cells in the microtiter plate or microwell array. An optional fluid delivery device 305 (see Figure 8) is controlled by the system to dispense fluids into the wells during the scan.

High throughput processing 306 is first performed on the microtiter plate or microwell array by acquiring and analyzing the signal from each of the wells in the plate. The processing performed in high throughput mode 307 is illustrated in Figure 12 and described below. Wells that exhibit some selected intensity response in this high throughput mode ("hits") are identified by the system. The system performs a conditional operation 308 that tests for hits. If hits are found, those specific hit wells are further analyzed in high content (micro level) mode 309. The processing performed in high content mode 312 is illustrated in Figure 13. The system then updates 310 the informatics database 311 with results of the measurements on the plate. If there are

more plates to be analyzed 313 the system loads the next plate 303; otherwise the analysis of the plates terminates 314.

The following discussion describes the high throughput mode illustrated in Figure 12. The preferred embodiment of the system, the single platform dual mode screening system, will be described. Those skilled in the art will recognize that operationally the dual platform system simply involves moving the plate between two optical systems rather than moving the optics. Once the system has been set up and the plate loaded, the system begins the HTS acquisition and analysis 401. The HTS optical module is selected by controlling a motorized optical positioning device 402 on the dual mode system. In one fluorescence channel, data from a primary marker on the plate is acquired 403 and wells are isolated from the plate background using a masking procedure 404. Images are also acquired in other fluorescence channels being used 405. The region in each image corresponding to each well 406 is measured 407. A feature calculated from the measurements for a particular well is compared with a predefined threshold or intensity response 408, and based on the result the well is either flagged as a "hit" 409 or not. The locations of the wells flagged as hits are recorded for subsequent high content mode processing. If there are wells remaining to be processed 410 the program loops back 406 until all the wells have been processed 411 and the system exits high throughput mode.

Following HTS analysis, the system starts the high content mode processing 501 defined in Figure 13. The system selects the HCS optical module 502 by controlling the motorized positioning system. For each "hit" well identified in high throughput mode, the XY stage location of the well is retrieved from memory or disk and the stage is then moved to the selected stage location 503. The autofocus procedure 504 is called for the first field in each hit well and then once every 5 to 8 fields within each well. In one channel, images are acquired of the primary marker 505 (typically cell nuclei counterstained with DAPI, Hoechst or PI fluorescent dye). The images are then segmented (separated into regions of nuclei and non-nuclei) using an adaptive thresholding procedure 506. The output of the segmentation procedure is a binary mask wherein the objects are white and the background is black. This binary image, also called a mask in the art, is used to determine if the field contains objects 507. The mask

is labeled with a blob labeling method whereby each object (or blob) has a unique number assigned to it. If objects are found in the field, images are acquired for all other active channels 508, otherwise the stage is advanced to the next field 514 in the current well. Each object is located in the image for further analysis 509. Morphological features, such as area and shape of the objects, are used to select objects likely to be cell nuclei 510, and discard (do no further processing on) those that are considered artifacts. For each valid cell nucleus, the XYZ stage location is recorded, a small image of the cell is stored, and assay specific features are measured 511. The system then performs multiple tests on the cells by applying several analytical methods to measure features at each of several wavelengths. After measuring the cell features, the systems checks if there are any unprocessed objects in the current field 512. If there are any unprocessed objects, it locates the next object 509 and determines whether it meets the criteria for a valid cell nucleus 510, and measures its features. After processing all the objects in the current field, the system determines whether it needs to find more cells or fields in the current well 513. If it needs to find more cells or fields in the current well it advances the XYZ stage to the next field within the current well 515. Otherwise, the system checks whether it has any remaining hit wells to measure 515. If so, it advances to the next hit well 503 and proceeds through another cycle of acquisition and analysis, otherwise the HCS mode is finished 516.

In an alternative embodiment of the present invention, a method of kinetic live cell screening is provided. The previously described embodiments of the invention are used to characterize the spatial distribution of cellular components at a specific point in time, the time of chemical fixation. As such, these embodiments have limited utility for implementing kinetic based screens, due to the sequential nature of the image acquisition, and the amount of time required to read all the wells on a plate. For example, since a plate can require 30 – 60 minutes to read through all the wells, only very slow kinetic processes can be measured by simply preparing a plate of live cells and then reading through all the wells more than once. Faster kinetic processes can be measured by taking multiple readings of each well before proceeding to the next well, but the elapsed time between the first and last well would be too long, and fast kinetic processes would likely be complete before reaching the last well.

The kinetic live cell extension of the invention enables the design and use of screens in which a biological process is characterized by its kinetics instead of, or in addition to, its spatial characteristics. In many cases, a response in live cells can be measured by adding a reagent to a specific well and making multiple measurements on that well with the appropriate timing. This dynamic live cell embodiment of the invention therefore includes apparatus for fluid delivery to individual wells of the system in order to deliver reagents to each well at a specific time in advance of reading the well. This embodiment thereby allows kinetic measurements to be made with temporal resolution of seconds to minutes on each well of the plate. To improve the overall efficiency of the dynamic live cell system, the acquisition control program is modified to allow repetitive data collection from sub-regions of the plate, allowing the system to read other wells between the time points required for an individual well.

Figure 8 describes an example of a fluid delivery device for use with the live cell embodiment of the invention and is described above. This set-up allows one set of pipette tips 705, or even a single pipette tip, to deliver reagent to all the wells on the plate. The bank of syringe pumps 701 can be used to deliver fluid to 12 wells simultaneously, or to fewer wells by removing some of the tips 705. The temporal resolution of the system can therefore be adjusted, without sacrificing data collection efficiency, by changing the number of tips and the scan pattern as follows. Typically, the data collection and analysis from a single well takes about 5 seconds. Moving from well to well and focusing in a well requires about 5 seconds, so the overall cycle time for a well is about 10 seconds. Therefore, if a single pipette tip is used to deliver fluid to a single well, and data is collected repetitively from that well, measurements can be made with about 5 seconds temporal resolution. If 6 pipette tips are used to deliver fluids to 6 wells simultaneously, and the system repetitively scans all 6 wells, each scan will require 60 seconds, thereby establishing the temporal resolution. For slower processes which only require data collection every 8 minutes, fluids can be delivered to one half of the plate, by moving the plate during the fluid delivery phase, and then repetitively scanning that half of the plate. Therefore, by adjusting the size of the sub-region being scanned on the plate, the temporal resolution can be adjusted without having to insert wait times between acquisitions. Because the system is continuously scanning and acquiring data, the overall time to collect a kinetic data set from the plate

is then simply the time to perform a single scan of the plate, multiplied by the number of time points required. Typically, 1 time point before addition of compounds and 2 or 3 time points following addition should be sufficient for screening purposes.

Figure 14 shows the acquisition sequence used for kinetic analysis. The start of processing 801 is configuration of the system, much of which is identical to the standard HCS configuration. In addition, the operator must enter information specific to the kinetic analysis being performed 802, such as the sub-region size, the number of time points required, and the required time increment. A sub-region is a group of wells that will be scanned repetitively in order to accumulate kinetic data. The size of the sub-region is adjusted so that the system can scan a whole sub-region once during a single time increment, thus minimizing wait times. The optimum sub-region size is calculated from the setup parameters, and adjusted if necessary by the operator. The system then moves the plate to the first sub-region 803, and to the first well in that sub-region 804 to acquire the prestimulation (time = 0) time points. The acquisition sequence performed in each well is exactly the same as that required for the specific HCS being run in kinetic mode. Figure 15 details a flow chart for that processing. All of the steps between the start 901 and the return 902 are identical to those described as steps 504 – 514 in Figure 13.

After processing each well in a sub-region, the system checks to see if all the wells in the sub-region have been processed 806 (Figure 14), and cycles through all the wells until the whole region has been processed. The system then moves the plate into position for fluid addition, and controls fluidic system delivery of fluids to the entire sub-region 807. This may require multiple additions for sub-regions which span several rows on the plate, with the system moving the plate on the X,Y stage between additions. Once the fluids have been added, the system moves to the first well in the sub-region 808 to begin acquisition of time points. The data is acquired from each well 809 and as before the system cycles through all the wells in the sub-region 810. After each pass through the sub-region, the system checks whether all the time points have been collected 811 and if not, pauses 813 if necessary 812 to stay synchronized with the requested time increment. Otherwise, the system checks for additional sub-regions on the plate 814 and either moves to the next sub-region 803 or finishes 815. Thus, the

kinetic analysis mode comprises operator identification of sub-regions of the microtiter plate or microwells to be screened, based on the kinetic response to be investigated, with data acquisitions within a sub-region prior to data acquisition in subsequent sub-regions.

5 *Specific Screens*

In another aspect of the present invention, cell screening methods and machine readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute procedures for defining the distribution and activity of specific cellular constituents and processes is provided. In a preferred
10 embodiment, the cell screening system comprises a high magnification fluorescence optical system with a stage adapted for holding cells and a means for moving the stage, a digital camera, a light source for receiving and processing the digital data from the digital camera, and a computer means for receiving and processing the digital data from the digital camera. This aspect of the invention comprises programs that instruct the
15 cell screening system to define the distribution and activity of specific cellular constituents and processes, using the luminescent probes, the optical imaging system, and the pattern recognition software of the invention. Preferred embodiments of the machine readable storage medium comprise programs consisting of a set of instructions for causing a cell screening system to execute the procedures set forth in Figures 9, 11,
20 12, 13, 14 or 15. Another preferred embodiment comprises a program consisting of a set of instructions for causing a cell screening system to execute procedures for detecting the distribution and activity of specific cellular constituents and processes. In most preferred embodiments, the cellular processes include, but are not limited to, nuclear translocation of a protein, cellular morphology, apoptosis, receptor
25 internalization, and protease-induced translocation of a protein.

In a preferred embodiment, the cell screening methods are used to identify compounds that modify the various cellular processes. The cells can be contacted with a test compound, and the effect of the test compound on a particular cellular process can be analyzed. Alternatively, the cells can be contacted with a test compound and a
30 known agent that modifies the particular cellular process, to determine whether the test compound can inhibit or enhance the effect of the known agent. Thus, the methods can

be used to identify test compounds that increase or decrease a particular cellular response, as well as to identify test compounds that affects the ability of other agents to increase or decrease a particular cellular response.

5 In another preferred embodiment, the locations containing cells are analyzed using the above methods at low resolution in a high throughput mode, and only a subset of the locations containing cells are analyzed in a high content mode to obtain luminescent signals from the luminescently labeled reporter molecules in subcellular compartments of the cells being analyzed.

10 The following examples are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined in the claims appended hereto.

The various chemical compounds, reagents, dyes, and antibodies that are referred to in the following Examples are commercially available from such sources as Sigma Chemical (St. Louis, MO), Molecular Probes (Eugene, OR), Aldrich Chemical
15 Company (Milwaukee, WI), Accurate Chemical Company (Westbury, NY), Jackson Immunolabs, and Clontech (Palo Alto, CA).

Example 1 **Cytoplasm to Nucleus Translocation Screening:**

a. Transcription Factors

20 Regulation of transcription of some genes involves activation of a transcription factor in the cytoplasm, resulting in that factor being transported into the nucleus where it can initiate transcription of a particular gene or genes. This change in transcription factor distribution is the basis of a screen for the cell-based screening system to detect compounds that inhibit or induce transcription of a particular gene or group of genes.
25 A general description of the screen is given followed by a specific example.

The distribution of the transcription factor is determined by labeling the nuclei with a DNA specific fluorophore like Hoechst 33423 and the transcription factor with a specific fluorescent antibody. After autofocusing on the Hoechst labeled nuclei, an image of the nuclei is acquired in the cell-based screening system and used to create a
30 mask by one of several optional thresholding methods, as described *supra*. The morphological descriptors of the regions defined by the mask are compared with the

user defined parameters and valid nuclear masks are identified and used with the following method to extract transcription factor distributions. Each valid nuclear mask is eroded to define a slightly smaller nuclear region. The original nuclear mask is then dilated in two steps to define a ring shaped region around the nucleus, which represents a cytoplasmic region. The average antibody fluorescence in each of these two regions is determined, and the difference between these averages is defined as the NucCyt Difference. Two examples of determining nuclear translocation are discussed below and illustrated in Figure 10A-J. Figure 10A illustrates an unstimulated cell with its nucleus 200 labeled with a blue fluorophore and a transcription factor in the cytoplasm 201 labeled with a green fluorophore. Figure 10B illustrates the nuclear mask 202 derived by the cell-based screening system. Figure 10C illustrates the cytoplasm 203 of the unstimulated cell imaged at a green wavelength. Figure 10D illustrates the nuclear mask 202 is eroded (reduced) once to define a nuclear sampling region 204 with minimal cytoplasmic distribution. The nucleus boundary 202 is dilated (expanded) several times to form a ring that is 2-3 pixels wide that is used to define the cytoplasmic sampling region 205 for the same cell. Figure 10E further illustrates a side view which shows the nuclear sampling region 204 and the cytoplasmic sampling region 205. Using these two sampling regions, data on nuclear translocation can be automatically analyzed by the cell-based screening system on a cell by cell basis. Figure 10F-J illustrates the strategy for determining nuclear translocation in a stimulated cell. Figure 10F illustrates a stimulated cell with its nucleus 206 labeled with a blue fluorophore and a transcription factor in the cytoplasm 207 labeled with a green fluorophore. The nuclear mask 208 in Figure 10G is derived by the cell based screening system. Figure 10H illustrates the cytoplasm 209 of a stimulated cell imaged at a green wavelength. Figure 10I illustrates the nuclear sampling region 211 and cytoplasmic sampling region 212 of the stimulated cell. Figure 10J further illustrates a side view which shows the nuclear sampling region 211 and the cytoplasmic sampling region 212.

A specific application of this method has been used to validate this method as a screen. A human cell line was plated in 96 well microtiter plates. Some rows of wells were titrated with IL-1, a known inducer of the NF-KB transcription factor. The cells were then fixed and stained by standard methods with a fluorescein labeled antibody to

the transcription factor, and Hoechst 33423. The cell-based screening system was used to acquire and analyze images from this plate and the NucCyt Difference was found to be strongly correlated with the amount of agonist added to the wells as illustrated in Figure 16. In a second experiment, an antagonist to the receptor for IL-1, IL-1RA was
5 titrated in the presence of IL-1 α , progressively inhibiting the translocation induced by IL-1 α . The NucCyt Difference was found to strongly correlate with this inhibition of translocation, as illustrated in **Figure 17**.

Additional experiments have shown that the NucCyt Difference, as well as the NucCyt ratio, gives consistent results over a wide range of cell densities and reagent
10 concentrations, and can therefore be routinely used to screen compound libraries for specific nuclear translocation activity. Furthermore, the same method can be used with antibodies to other transcription factors, or GFP-transcription factor chimeras, or fluorescently labeled transcription factors introduced into living or fixed cells, to screen for effects on the regulation of transcription factor activity.

Figure 18 is a representative display on a PC screen of data which was obtained in accordance with Example 1. Graph 1 180 plots the difference between the average antibody fluorescence in the nuclear sampling region and cytoplasmic sampling region, NucCyt Difference versus Well #. Graph 2 181 plots the average fluorescence of the antibody in the nuclear sampling region, NP1 average, versus the Well #. Graph 3 182
20 plots the average antibody fluorescence in the cytoplasmic sampling region, LIP1 average, versus Well #. The software permits displaying data from each cell. For example, **Figure 18** shows a screen display 183, the nuclear image 184, and the fluorescent antibody image 185 for cell #26.

NucCyt Difference referred to in graph 1 180 of **Figure 18** is the difference
25 between the average cytoplasmic probe (fluorescent reporter molecule) intensity and the average nuclear probe (fluorescent reporter molecule) intensity. NP1 average referred to in graph 2 181 of **Figure 18** is the average of cytoplasmic probe (fluorescent reporter molecule) intensity within the nuclear sampling region. LIP1 average referred to in graph 3 182 of **Figure 18** is the average probe (fluorescent reporter molecule)
30 intensity within the cytoplasmic sampling region.

It will be understood by one of skill in the art that this aspect of the invention can be performed using other transcription factors that translocate from the cytoplasm

to the nucleus upon activation. In another specific example, activation of the c-fos transcription factor was assessed by defining its spatial position within cells. Activated c-fos is found only within the nucleus, while inactivated c-fos resides within the cytoplasm.

5 3T3 cells were plated at 5000-10000 cells per well in a Polyfiltronics 96-well plate. The cells were allowed to attach and grow overnight. The cells were rinsed twice with 100 μ l serum-free medium, incubated for 24-30 hours in serum-free MEM culture medium, and then stimulated with platelet derived growth factor (PDGF-BB) (Sigma Chemical Co., St. Louis, MO) diluted directly into serum free medium at
10 concentrations ranging from 1-50 ng/ml for an average time of 20 minutes.

Following stimulation, cells were fixed for 20 minutes in 3.7% formaldehyde solution in 1X Hanks buffered saline solution (HBSS). After fixation, the cells were washed with HBSS to remove residual fixative, permeabilized for 90 seconds with 0.5% Triton X-100 solution in HBSS, and washed twice with HBSS to remove residual
15 detergent. The cells were then blocked for 15 minutes with a 0.1% solution of BSA in HBSS, and further washed with HBSS prior to addition of diluted primary antibody solution.

c-Fos rabbit polyclonal antibody (Calbiochem, PC05) was diluted 1:50 in HBSS, and 50 μ l of the dilution was applied to each well. Cells were incubated in the
20 presence of primary antibody for one hour at room temperature, and then incubated for one hour at room temperature in a light tight container with goat anti-rabbit secondary antibody conjugated to ALEXATM 488 (Molecular Probes), diluted 1:500 from a 100 μ g/ml stock in HBSS. Hoechst DNA dye (Molecular Probes) was then added at a 1:1000 dilution of the manufacturer's stock solution (10 mg/ml). The cells were then
25 washed with HBSS, and the plate was sealed prior to analysis with the cell screening system of the invention. The data from these experiments demonstrated that the methods of the invention could be used to measure transcriptional activation of c-fos by defining its spatial position within cells.

One of skill in the art will recognize that while the following method is applied to
30 detection of c-fos activation, it can be applied to the analysis of any transcription factor that translocates from the cytoplasm to the nucleus upon activation. Examples of such transcription factors include, but are not limited to fos and jun homologs, NF-KB

(nuclear factor kappa from B cells), NFAT (nuclear factor of activated T-lymphocytes), and STATs (signal transducer and activator of transcription) factors (For example, see Strehlow, I., and Schindler, C. 1998. *J. Biol. Chem.* 273:28049-28056; Chow, et al. 1997 *Science*. 278:1638-1641; Ding et al. 1998 *J. Biol. Chem.* 273:28897-28905; Baldwin, 1996. *Annu Rev Immunol.* 14:649-83; Kuo, C.T., and J.M. Leiden. 1999. *Annu Rev Immunol.* 17:149-87; Rao, et al. 1997. *Annu Rev Immunol.* 15:707-47; Masuda, et al. 1998. *Cell Signal.* 10:599-611; Hoey, T., and U. Schindler. 1998. *Curr Opin Genet Dev.* 8:582-7; Liu, et al. 1998. *Curr Opin Immunol.* 10:271-8.)

Thus, in this aspect of the invention, indicator cells are treated with test compounds and the distribution of luminescently labeled transcription factor is measured in space and time using a cell screening system, such as the one disclosed above. The luminescently labeled transcription factor may be expressed by or added to the cells either before, together with, or after contacting the cells with a test compound.

For example, the transcription factor may be expressed as a luminescently labeled protein chimera by transfected indicator cells. Alternatively, the luminescently labeled transcription factor may be expressed, isolated, and bulk-loaded into the indicator cells as described above, or the transcription factor may be luminescently labeled after isolation. As a further alternative, the transcription factor is expressed by the indicator cell, which is subsequently contacted with a luminescent label, such as an antibody, that detects the transcription factor.

In a further aspect, kits are provided for analyzing transcription factor activation, comprising an antibody that specifically recognizes a transcription factor of interest, and instructions for using the antibody for carrying out the methods described above. In a preferred embodiment, the transcription factor-specific antibody, or a secondary antibody that detects the transcription factor antibody, is luminescently labeled. In further preferred embodiments, the kit contains cells that express the transcription factor of interest, and/or the kit contains a compound that is known to modify activation of the transcription factor of interest, including but not limited to platelet derived growth factor (PDGF) and serum, which both modify fos activation; and interleukin 1(IL-1) and tumor necrosis factor (TNF), which both modify NF-KB activation.

In another embodiment, the kit comprises a recombinant expression vector comprising a nucleic acid encoding a transcription factor of interest that translocates

from the cytoplasm to the nucleus upon activation, and instructions for using the expression vector to identify compounds that modify transcription factor activation in a cell of interest. Alternatively, the kits contain a purified, luminescently labeled transcription factor. In a preferred embodiment, the transcription factor is expressed as a fusion protein with a luminescent protein, including but not limited to green fluorescent protein, luciferase, or mutants or fragments thereof. In various preferred embodiments, the kit further contains cells that are transfected with the expression vector, an antibody or fragment that specifically bind to the transcription factor of interest, and/or a compound that is known to modify activation of the transcription factor of interest (as above).

b. Protein Kinases

The cytoplasm to nucleus screening methods can also be used to analyze the activation of any protein kinase that is present in an inactive state in the cytoplasm and is transported to the nucleus upon activation, or that phosphorylates a substrate that translocates from the cytoplasm to the nucleus upon phosphorylation. Examples of appropriate protein kinases include, but are not limited to extracellular signal-regulated protein kinases (ERKs), c-Jun amino-terminal kinases (JNKs), Fos regulating protein kinases (FRKs), p38 mitogen activated protein kinase (p38MAPK), protein kinase A (PKA), and mitogen activated protein kinase kinases (MAPKKs). (For example, see Hall, et al. 1999. *J Biol Chem.* 274:376-83; Han, et al. 1995. *Biochim. Biophys. Acta.* 1265:224-227; Jaaro et al. 1997. *Proc. Natl. Acad. Sci. U.S.A.* 94:3742-3747; Taylor, et al. 1994. *J. Biol. Chem.* 269:308-318; Zhao, Q., and F. S. Lee. 1999. *J Biol Chem.* 274:8355-8; Paolillo et al. 1999. *J Biol Chem.* 274:6546-52; Coso et al. 1995. *Cell* 81:1137-1146; Tibbles, L.A., and J.R. Woodgett. 1999. *Cell Mol Life Sci.* 55:1230-54; Schaeffer, H.J., and M.J. Weber. 1999. *Mol Cell Biol.* 19:2435-44.)

Alternatively, protein kinase activity is assayed by monitoring translocation of a luminescently labeled protein kinase substrate from the cytoplasm to the nucleus after being phosphorylated by the protein kinase of interest. In this embodiment, the substrate is non-phosphorylated and cytoplasmic prior to phosphorylation, and is translocated to the nucleus upon phosphorylation by the protein kinase. There is no requirement that the protein kinase itself translocates from the cytoplasm to the nucleus

in this embodiment. Examples of such substrates (and the corresponding protein kinase) include, but are not limited to c-jun (JNK substrate); fos (FRK substrate), and p38 (p38 MAPK substrate).

Thus, in these embodiments, indicator cells are treated with test compounds and the distribution of luminescently labeled protein kinase or protein kinase substrate is measured in space and time using a cell screening system, such as the one disclosed above. The luminescently labeled protein kinase or protein kinase substrate may be expressed by or added to the cells either before, together with, or after contacting the cells with a test compound. For example, the protein kinase or protein kinase substrate may be expressed as a luminescently labeled protein chimera by transfected indicator cells. Alternatively, the luminescently labeled protein kinase or protein kinase substrate may be expressed, isolated, and bulk-loaded into the indicator cells as described above, or the protein kinase or protein kinase substrate may be luminescently labeled after isolation. As a further alternative, the protein kinase or protein kinase substrate is expressed by the indicator cell, which is subsequently contacted with a luminescent label, such as a labeled antibody, that detects the protein kinase or protein kinase substrate.

In a further embodiment, protein kinase activity is assayed by monitoring the phosphorylation state (ie: phosphorylated or not phosphorylated) of a protein kinase substrate. In this embodiment, there is no requirement that either the protein kinase or the protein kinase substrate translocate from the cytoplasm to the nucleus upon activation. In a preferred embodiment, phosphorylation state is monitored by contacting the cells with an antibody that binds only to the phosphorylated form of the protein kinase substrate of interest (For example, as disclosed in U.S. Patent No. 5,599,681).

In another preferred embodiment, a biosensor of phosphorylation is used. For example, a luminescently labeled protein or fragment thereof can be fused to a protein that has been engineered to contain (a) a phosphorylation site that is recognized by a protein kinase of interest; and (b) a nuclear localization signal that is unmasked by the phosphorylation. Such a biosensor will thus be translocated to the nucleus upon phosphorylation, and its translocation can be used as a measure of protein kinase activation.

In another aspect, kits are provided for analyzing protein kinase activation, comprising a primary antibody that specifically binds to a protein kinase, a protein kinase substrate, or a phosphorylated form of the protein kinase substrate of interest and instructions for using the primary antibody to identify compounds that modify protein kinase activation in a cell of interest. In a preferred embodiment, the primary antibody, or a secondary antibody that detects the primary antibody, is luminescently labeled. In other preferred embodiments, the kit further comprises cells that express the protein kinase of interest, and/or a compound that is known to modify activation of the protein kinase of interest, including but not limited to dibutyryl cAMP (modifies PKA), forskolin (PKA), and anisomycin (p38MAPK).

Alternatively, the kits comprise an expression vector encoding a protein kinase or a protein kinase substrate of interest that translocates from the cytoplasm to the nucleus upon activation and instructions for using the expression vector to identify compounds that modify protein kinase activation in a cell of interest. Alternatively, the kits contain a purified, luminescently labeled protein kinase or protein kinase substrate. In a preferred embodiment, the protein kinase or protein kinase substrate of interest is expressed as a fusion protein with a luminescent protein. In further preferred embodiments, the kit further comprises cells that are transfected with the expression vector, an antibody or fragment thereof that specifically binds to the protein kinase or protein kinase substrate of interest, and/or a compound that is known to modify activation of the protein kinase of interest. (as above)

In another aspect, the present invention comprises a machine readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute the methods disclosed for analyzing transcription factor or protein kinase activation, wherein the cell screening system comprises an optical system with a stage adapted for holding a plate containing cells, a digital camera, a means for directing fluorescence or luminescence emitted from the cells to the digital camera, and a computer means for receiving and processing the digital data from the digital camera.

Example 2 Automated Screen for Compounds that Modify Cellular Morphology

Changes in cell size are associated with a number of cellular conditions, such as hypertrophy, cell attachment and spreading, differentiation, growth and division, necrotic and programmed cell death, cell motility, morphogenesis, tube formation, and colony formation.

For example, cellular hypertrophy has been associated with a cascade of alterations in gene expression and can be characterized in cell culture by an alteration in cell size, that is clearly visible in adherent cells growing on a coverslip.

Cell size can also be measured to determine the attachment and spreading of adherent cells. Cell spreading is the result of selective binding of cell surface receptors to substrate ligands and subsequent activation of signaling pathways to the cytoskeleton. Cell attachment and spreading to substrate molecules is an important step for the metastasis of cancer cells, leukocyte activation during the inflammatory response, keratinocyte movement during wound healing, and endothelial cell movement during angiogenesis. Compounds that affect these surface receptors, signaling pathways, or the cytoskeleton will affect cell spreading and can be screened by measuring cell size.

Total cellular area can be monitored by labeling the entire cell body or the cell cytoplasm using cytoskeletal markers, cytosolic volume markers, or cell surface markers, in conjunction with a DNA label. Examples of such labels (many available from Molecular Probes (Eugene, Oregon) and Sigma Chemical Co. (St. Louis, Missouri)) include the following:

CELL SIZE AND AREA MARKERS
Cytoskeletal Markers
• ALEXA TM 488 phalloidin (Molecular Probes, Oregon)
• Tubulin-green fluorescent protein chimeras
• Cytokeratin-green fluorescent protein chimeras
• Antibodies to cytoskeletal proteins
Cytosolic Volume Markers
• Green fluorescent proteins
• Chloromethylfluorescein diacetate (CMFDA)
• Calcein green
• BCECF/AM ester
• Rhodamine dextran
Cell Surface Markers for Lipid, Protein, or Oligosaccharide
• Dihexadecyl tetramethylindocarbocyanine perchlorate (DiIC16) lipid dyes
• Triethylammonium propyl dibutylamino styryl pyridinium (FM 4-64, FM 1-43) lipid dyes
• MITOTRACKER TM Green FM
• Lectins to oligosaccharides such as fluorescein concanavalin A or wheat germ agglutinin
• SYPRO TM Red non-specific protein markers
• Antibodies to various surface proteins such as epidermal growth factor
• Biotin labeling of surface proteins followed by fluorescent streptavidin labeling

Protocols for cell staining with these various agents are well known to those skilled in the art. Cells are stained live or after fixation and the cell area can be measured. For example, live cells stained with DiIC16 have homogeneously labeled plasma membranes, and the projected cross-sectional area of the cell is uniformly discriminated from background by fluorescence intensity of the dye. Live cells stained with cytosolic stains such as CMFDA produce a fluorescence intensity that is proportional to cell thickness. Although cell labeling is dimmer in thin regions of the cell, total cell area can be discriminated from background. Fixed cells can be stained with cytoskeletal markers such as ALEXATM 488 phalloidin that label polymerized actin. Phalloidin does not homogeneously stain the cytoplasm, but still permits discrimination of the total cell area from background.

Cellular hypertrophy

A screen to analyze cellular hypertrophy is implemented using the following strategy. Primary rat myocytes can be cultured in 96 well plates, treated with various compounds and then fixed and labeled with a fluorescent marker for the cell membrane or cytoplasm, or cytoskeleton, such as an antibody to a cell surface marker or a

fluorescent marker for the cytoskeleton like rhodamine-phalloidin, in combination with a DNA label like Hoechst.

After focusing on the Hoechst labeled nuclei, two images are acquired, one of the Hoechst labeled nuclei and one of the fluorescent cytoplasm image. The nuclei are identified by thresholding to create a mask and then comparing the morphological descriptors of the mask with a set of user defined descriptor values. Each non-nucleus image (or "cytoplasmic image") is then processed separately. The original cytoplasm image can be thresholded, creating a cytoplasmic mask image. Local regions containing cells are defined around the nuclei. The limits of the cells in those regions are then defined by a local dynamic threshold operation on the same region in the fluorescent antibody image. A sequence of erosions and dilations is used to separate slightly touching cells and a second set of morphological descriptors is used to identify single cells. The area of the individual cells is tabulated in order to define the distribution of cell sizes for comparison with size data from normal and hypertrophic cells.

Responses from entire 96-well plates (measured as average cytoplasmic area/cell) were analyzed by the above methods, and the results demonstrated that the assay will perform the same on a well-to-well, plate-to-plate, and day-to-day basis (below a 15% cov for maximum signal). The data showed very good correlation for each day, and that there was no variability due to well position in the plate.

The following totals can be computed for the field. The aggregate whole nucleus area is the number of nonzero pixels in the nuclear mask. The average whole nucleus area is the aggregate whole nucleus area divided by the total number of nuclei. For each cytoplasm image several values can be computed. These are the total cytoplasmic area, which is the count of nonzero pixels in the cytoplasmic mask. The aggregate cytoplasm intensity is the sum of the intensities of all pixels in the cytoplasmic mask. The cytoplasmic area per nucleus is the total cytoplasmic area divided by the total nucleus count. The cytoplasmic intensity per nucleus is the aggregate cytoplasm intensity divided by the total nucleus count. The average cytoplasm intensity is the aggregate cytoplasm intensity divided by the cytoplasm area. The cytoplasm nucleus ratio is the total cytoplasm area divided by the total nucleus area.

Additionally, one or more fluorescent antibodies to other cellular proteins, such as the major muscle proteins actin or myosin, can be included. Images of these additional labeled proteins can be acquired and stored with the above images, for later review, to identify anomalies in the distribution and morphology of these proteins in hypertrophic cells. This example of a multi-parametric screen allows for simultaneous analysis of cellular hypertrophy and changes in actin or myosin distribution.

One of skill in the art will recognize that while the example analyzes myocyte hypertrophy, the methods can be applied to analyzing hypertrophy, or general morphological changes in any cell type.

Cell morphology assays for prostate carcinoma

Cell spreading is a measure of the response of cell surface receptors to substrate attachment ligands. Spreading is proportional to the ligand concentration or to the concentration of compounds that reduce receptor-ligand function. One example of selective cell-substrate attachment is prostate carcinoma cell adhesion to the extracellular matrix protein collagen. Prostate carcinoma cells metastasize to bone via selective adhesion to collagen.

Compounds that interfere with metastasis of prostate carcinoma cells were screened as follows. PC3 human prostate carcinoma cells were cultured in media with appropriate stimulants and are passaged to collagen coated 96 well plates. Ligand concentration can be varied or inhibitors of cell spreading can be added to the wells. Examples of compounds that can affect spreading are receptor antagonists such as integrin- or proteoglycan-blocking antibodies, signaling inhibitors including phosphatidyl inositol-3 kinase inhibitors, and cytoskeletal inhibitors such as cytochalasin D. After two hours, cells were fixed and stained with ALEXATM 488 phalloidin (Molecular Probes) and Hoechst 33342 as per the protocol for cellular hypertrophy. The size of cells under these various conditions, as measured by cytoplasmic staining, can be distinguished above background levels. The number of cells per field is determined by measuring the number of nuclei stained with the Hoechst DNA dye. The area per cell is found by dividing the cytoplasmic area (phalloidin image) by the cell number (Hoechst image). The size of cells is proportional to the ligand-receptor function. Since the area is determined by ligand

concentration and by the resultant function of the cell, drug efficacy, as well as drug potency, can be determined by this cell-based assay. Other measurements can be made as discussed above for cellular hypertrophy.

The methods for analyzing cellular morphology can be used in a combined high
5 throughput-high content screen. In one example, the high throughput mode scans the whole well for an increase in fluorescent phalloidin intensity. A threshold is set above which both nuclei (Hoechst) and cells (phalloidin) are measured in a high content mode. In another example, an environmental biosensor (examples include, but are not limited to, those biosensors that are sensitive to calcium and pH changes) is added to
10 the cells, and the cells are contacted with a compound. The cells are scanned in a high throughput mode, and those wells that exceed a pre-determined threshold for luminescence of the biosensor are scanned in a high content mode.

In a further aspect, kits are provided for analyzing cellular morphology, comprising a luminescent compound that can be used to specifically label the cell
15 cytoplasm, membrane, or cytoskeleton (such as those described above), and instructions for using the luminescent compound to identify test stimuli that induce or inhibit changes in cellular morphology according to the above methods. In a preferred embodiment, the kit further comprises a luminescent marker for cell nuclei. In a further preferred embodiment, the kit comprises at least one compound that is known to
20 modify cellular morphology, including, but not limited to integrin- or proteoglycan-blocking antibodies, signaling inhibitors including phosphatidyl inositol-3 kinase inhibitors, and cytoskeletal inhibitors such as cytochalasin D.

In another aspect, the present invention comprises a machine readable storage medium comprising a program containing a set of instructions for causing a cell
25 screening system to execute the disclosed methods for analyzing cellular morphology, wherein the cell screening system comprises an optical system with a stage adapted for holding a plate containing cells, a digital camera, a means for directing fluorescence or luminescence emitted from the cells to the digital camera, and a computer means for receiving and processing the digital data from the digital camera.

30 *Example 3 Dual Mode High Throughput and High-Content Screen*

The following example is a screen for activation of a G-protein coupled receptor (GPCR) as detected by the translocation of the GPCR from the plasma membrane to a

proximal nuclear location. This example illustrates how a high throughput screen can be coupled with a high-content screen in the dual mode System for Cell Based Screening.

G-protein coupled receptors are a large class of 7 trans-membrane domain cell surface receptors. Ligands for these receptors stimulate a cascade of secondary signals in the cell, which may include, but are not limited to, Ca^{++} transients, cyclic AMP production, inositol triphosphate (IP_3) production and phosphorylation. Each of these signals are rapid, occurring in a matter of seconds to minutes, but are also generic. For example, many different GPCRs produce a secondary Ca^{++} signal when activated. Stimulation of a GPCR also results in the transport of that GPCR from the cell surface membrane to an internal, proximal nuclear compartment. This internalization is a much more receptor-specific indicator of activation of a particular receptor than are the secondary signals described above.

Figure 19 illustrates a dual mode screen for activation of a GPCR. Cells carrying a stable chimera of the GPCR with a blue fluorescent protein (BFP) would be loaded with the acetoxymethylester form of Fluo-3, a cell permeable calcium indicator (green fluorescence) that is trapped in living cells by the hydrolysis of the esters. They would then be deposited into the wells of a microtiter plate 601. The wells would then be treated with an array of test compounds using a fluid delivery system, and a short sequence of Fluo-3 images of the whole microtiter plate would be acquired and analyzed for wells exhibiting a calcium response (i.e., high throughput mode). The images would appear like the illustration of the microtiter plate 601 in Figure 19. A small number of wells, such as wells C4 and E9 in the illustration, would fluoresce more brightly due to the Ca^{++} released upon stimulation of the receptors. The locations of wells containing compounds that induced a response 602, would then be transferred to the HCS program and the optics switched for detailed cell by cell analysis of the blue fluorescence for evidence of GPCR translocation to the perinuclear region. The bottom of Figure 19 illustrates the two possible outcomes of the analysis of the high resolution cell data. The camera images a sub-region 604 of the well area 603, producing images of the fluorescent cells 605. In well C4, the uniform distribution of the fluorescence in the cells indicates that the receptor has not internalized, implying that the Ca^{++} response

seen was the result of the stimulation of some other signalling system in the cell. The cells in well E9 606 on the other hand, clearly indicate a concentration of the receptor in the perinuclear region clearly indicating the full activation of the receptor. Because only a few hit wells have to be analyzed with high resolution, the overall throughput of the dual mode system can be quite high, comparable to the high throughput system alone.

Example 4 Kinetic High Content Screen

The following is an example of a screen to measure the kinetics of internalization of a receptor. As described above, the stimulation of a GPCR, results in the internalization of the receptor, with a time course of about 15 min. Simply detecting the endpoint as internalized or not, may not be sufficient for defining the potency of a compound as a GPCR agonist or antagonist. However, 3 time points at 5 min intervals would provide information not only about potency during the time course of measurement, but would also allow extrapolation of the data to much longer time periods. To perform this assay, the sub-region would be defined as two rows, the sampling interval as 5 minutes and the total number of time points 3. The system would then start by scanning two rows, and then adding reagent to the two rows, establishing the time=0 reference. After reagent addition, the system would again scan the two row sub-region acquiring the first time point data. Since this process would take about 250 seconds, including scanning back to the beginning of the sub-region, the system would wait 50 seconds to begin acquisition of the second time point. Two more cycles would produce the three time points and the system would move on to the second 2 row sub-region. The final two 2-row sub-regions would be scanned to finish all the wells on the plate, resulting in four time points for each well over the whole plate. Although the time points for the wells would be offset slightly relative to time=0, the spacing of the time points would be very close to the required 5 minutes, and the actual acquisition times and results recorded with much greater precision than in a fixed-cell screen.

Example 5 High-content screen of human glucocorticoid receptor translocation

One class of HCS involves the drug-induced dynamic redistribution of intracellular constituents. The human glucocorticoid receptor (hGR), a single “sensor” in the complex environmental response machinery of the cell, binds steroid molecules that have diffused into the cell. The ligand-receptor complex translocates to the nucleus where transcriptional activation occurs (Htun et al., *Proc. Natl. Acad. Sci.* 93:4845, 1996).

In general, hormone receptors are excellent drug targets because their activity lies at the apex of key intracellular signaling pathways. Therefore, a high-content screen of hGR translocation has distinct advantage over *in vitro* ligand-receptor binding assays. The availability of up to two more channels of fluorescence in the cell screening system of the present invention permits the screen to contain two additional parameters in parallel, such as other receptors, other distinct targets or other cellular processes.

Plasmid construct. A eukaryotic expression plasmid containing a coding sequence for a green fluorescent protein – human glucocorticoid receptor (GFP-hGR) chimera was prepared using GFP mutants (Palm et al., *Nat. Struct. Biol.* 4:361 (1997)). The construct was used to transfect a human cervical carcinoma cell line (HeLa).

Cell preparation and transfection. HeLa cells (ATCC CCL-2) were trypsinized and plated using DMEM containing 5% charcoal/dextran-treated fetal bovine serum (FBS) (HyClone) and 1% penicillin-streptomycin (C-DMEM) 12-24 hours prior to transfection and incubated at 37°C and 5% CO₂. Transfections were performed by calcium phosphate co-precipitation (Graham and Van der Eb, *Virology* 52:456, 1973; Sambrook et al., (1989). *Molecular Cloning: A Laboratory Manual*, Second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) or with Lipofectamine (Life Technologies, Gaithersburg, MD). For the calcium phosphate transfections, the medium was replaced, prior to transfection, with DMEM containing 5% charcoal/dextran-treated FBS. Cells were incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C and 5% CO₂, washed 3-4 times with DMEM to remove the precipitate, followed by the addition of C-DMEM.

Lipofectamine transfections were performed in serum-free DMEM without antibiotics according to the manufacturer's instructions (Life Technologies,

Gaithersburg, MD). Following a 2-3 hour incubation with the DNA-liposome complexes, the medium was removed and replaced with C-DMEM. All transfected cells in 96-well microtiter plates were incubated at 33°C and 5% CO₂ for 24-48 hours prior to drug treatment. Experiments were performed with the receptor expressed transiently in HeLa cells.

Dexamethasone induction of GFP-hGR translocation. To obtain receptor-ligand translocation kinetic data, nuclei of transfected cells were first labeled with 5 µg/ml Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 33°C and 5% CO₂. Cells were washed once in Hank's Balanced Salt Solution (HBSS) followed by the addition of 100 nM dexamethasone in HBSS with 1% charcoal/dextran-treated FBS. To obtain fixed time point dexamethasone titration data, transfected HeLa cells were first washed with DMEM and then incubated at 33°C and 5% CO₂ for 1 h in the presence of 0 – 1000 nM dexamethasone in DMEM containing 1% charcoal/dextran-treated FBS. Cells were analyzed live or they were rinsed with HBSS, fixed for 15 min with 3.7% formaldehyde in HBSS, stained with Hoechst 33342, and washed before analysis. The intracellular GFP-hGR fluorescence signal was not diminished by this fixation procedure.

Image acquisition and analysis. Kinetic data were collected by acquiring fluorescence image pairs (GFP-hGR and Hoechst 33342-labeled nuclei) from fields of living cells at 1 min intervals for 30 min after the addition of dexamethasone. Likewise, image pairs were obtained from each well of the fixed time point screening plates 1 h after the addition of dexamethasone. In both cases, the image pairs obtained at each time point were used to define nuclear and cytoplasmic regions in each cell. Translocation of GFP-hGR was calculated by dividing the integrated fluorescence intensity of GFP-hGR in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. In the fixed time point screen this translocation ratio was calculated from data obtained from at least 200 cells at each concentration of dexamethasone tested. Drug-induced translocation of GFP-hGR from the cytoplasm to the nucleus was therefore correlated with an increase in the translocation ratio.

Results. Figure 20 schematically displays the drug-induced cytoplasm to nucleus translocation of the human glucocorticoid receptor. The upper pair of

schematic diagrams depicts the localization of GFP-hGR within the cell before 250 (A) and after 251 (B) stimulation with dexamethasone. Under these experimental conditions, the drug induces a large portion of the cytoplasmic GFP-hGR to translocate into the nucleus. This redistribution is quantified by determining the integrated intensities ratio of the cytoplasmic and nuclear fluorescence in treated 255 and untreated 254 cells. The lower pair of fluorescence micrographs show the dynamic redistribution of GFP-hGR in a single cell, before 254 and after 255 treatment. The HCS is performed on wells containing hundreds to thousands of transfected cells and the translocation is quantified for each cell in the field exhibiting GFP fluorescence. Although the use of a stably transfected cell line would yield the most consistently labeled cells, the heterogeneous levels of GFP-hGR expression induced by transient transfection did not interfere with analysis by the cell screening system of the present invention.

To execute the screen, the cell screening system scans each well of the plate, images a population of cells in each, and analyzes cells individually. Here, two channels of fluorescence are used to define the cytoplasmic and nuclear distribution of the GFP-hGR within each cell. Depicted in Figure 21 is the graphical user interface of the cell screening system near the end of a GFP-hGR screen. The user interface depicts the parallel data collection and analysis capability of the system. The windows labeled "Nucleus" 261 and "GFP-hGR" 262 show the pair of fluorescence images being obtained and analyzed in a single field. The window labeled "Color Overlay" 260 is formed by pseudocoloring the above images and merging them so the user can immediately identify cellular changes. Within the "Stored Object Regions" window 265, an image containing each analyzed cell and its neighbors is presented as it is archived. Furthermore, as the HCS data are being collected, they are analyzed, in this case for GFP-hGR translocation, and translated into an immediate "hit" response. The 96 well plate depicted in the lower window of the screen 267 shows which wells have met a set of user-defined screening criteria. For example, a white-colored well 269 indicates that the drug-induced translocation has exceeded a predetermined threshold value of 50%. On the other hand, a black-colored well 270 indicates that the drug being tested induced less than 10% translocation. Gray-colored wells 268 indicate "hits" where the translocation value fell between 10% and 50%. Row "E" on the 96 well

plate being analyzed 266 shows a titration with a drug known to activate GFP-hGR translocation, dexamethasone. This example screen used only two fluorescence channels. Two additional channels (Channels 3 263 and 4 264) are available for parallel analysis of other specific targets, cell processes, or cytotoxicity to create multiple parameter screens.

There is a link between the image database and the information database that is a powerful tool during the validation process of new screens. At the completion of a screen, the user has total access to image and calculated data (Figure 22). The comprehensive data analysis package of the cell screening system allows the user to examine HCS data at multiple levels. Images 276 and detailed data in a spread sheet 279 for individual cells can be viewed separately, or summary data can be plotted. For example, the calculated results of a single parameter for each cell in a 96 well plate are shown in the panel labeled Graph 1 275. By selecting a single point in the graph, the user can display the entire data set for a particular cell that is recalled from an existing database. Shown here are the image pair 276 and detailed fluorescence and morphometric data from a single cell (Cell #118, gray line 277). The large graphical insert 278 shows the results of dexamethasone concentration on the translocation of GFP-hGR. Each point is the average of data from at least 200 cells. The calculated EC₅₀ for dexamethasone in this assay is 2 nM.

A powerful aspect of HCS with the cell screening system is the capability of kinetic measurements using multicolor fluorescence and morphometric parameters in living cells. Temporal and spatial measurements can be made on single cells within a population of cells in a field. Figure 23 shows kinetic data for the dexamethasone-induced translocation of GFP-hGR in several cells within a single field. Human HeLa cells transfected with GFP-hGR were treated with 100 nM dexamethasone and the translocation of GFP-hGR was measured over time in a population of single cells. The graph shows the response of transfected cells 285, 286, 287, and 288 and non-transfected cells 289. These data also illustrate the ability to analyze cells with different expression levels.

Example 6 High-content screen of drug-induced apoptosis

Apoptosis is a complex cellular program that involves myriad molecular events and pathways. To understand the mechanisms of drug action on this process, it is essential to measure as many of these events within cells as possible with temporal and spatial resolution. Therefore, an apoptosis screen that requires little cell sample preparation yet provides an automated readout of several apoptosis-related parameters would be ideal. A cell-based assay designed for the cell screening system has been used to simultaneously quantify several of the morphological, organellar, and macromolecular hallmarks of paclitaxel-induced apoptosis.

Cell preparation. The cells chosen for this study were mouse connective tissue fibroblasts (L-929; ATCC CCL-1) and a highly invasive glioblastoma cell line (SNB-19; ATCC CRL-2219) (Welch et al., *In Vitro Cell. Dev. Biol.* 31:610, 1995). The day before treatment with an apoptosis inducing drug, 3500 cells were placed into each well of a 96-well plate and incubated overnight at 37°C in a humidified 5% CO₂ atmosphere. The following day, the culture medium was removed from each well and replaced with fresh medium containing various concentrations of paclitaxel (0 – 50 µM) from a 20 mM stock made in DMSO. The maximal concentration of DMSO used in these experiments was 0.25%. The cells were then incubated for 26 h as above. At the end of the paclitaxel treatment period, each well received fresh medium containing 750 nM MitoTracker Red (Molecular Probes; Eugene, OR) and 3 µg/ml Hoechst 33342 DNA-binding dye (Molecular Probes) and was incubated as above for 20 min. Each well on the plate was then washed with HBSS and fixed with 3.7% formaldehyde in HBSS for 15 min at room temperature. The formaldehyde was washed out with HBSS and the cells were permeabilized for 90 s with 0.5% (v/v) Triton X-100, washed with HBSS, incubated with 2 U ml⁻¹ Bodipy FL phalloidin (Molecular Probes) for 30 min, and washed with HBSS. The wells on the plate were then filled with 200 µl HBSS, sealed, and the plate stored at 4°C if necessary. The fluorescence signals from plates stored this way were stable for at least two weeks after preparation. As in the nuclear translocation assay, fluorescence reagents can be designed to convert this assay into a live cell high-content screen.

Image acquisition and analysis on the ArrayScan System. The fluorescence intensity of intracellular MitoTracker Red, Hoechst 33342, and Bodipy FL phalloidin

was measured with the cell screening system as described *supra*. Morphometric data from each pair of images obtained from each well was also obtained to detect each object in the image field (e.g., cells and nuclei), and to calculate its size, shape, and integrated intensity.

5 **Calculations and output.** A total of 50-250 cells were measured per image field. For each field of cells, the following calculations were performed: (1) The average nuclear area (μm^2) was calculated by dividing the total nuclear area in a field by the number of nuclei detected. (2) The average nuclear perimeter (μm) was calculated by dividing the sum of the perimeters of all nuclei in a field by the number
10 of nuclei detected in that field. Highly convoluted apoptotic nuclei had the largest nuclear perimeter values. (3) The average nuclear brightness was calculated by dividing the integrated intensity of the entire field of nuclei by the number of nuclei in that field. An increase in nuclear brightness was correlated with increased DNA content. (4) The average cellular brightness was calculated by dividing the integrated intensity of an
15 entire field of cells stained with MitoTracker dye by the number of nuclei in that field. Because the amount of MitoTracker dye that accumulates within the mitochondria is proportional to the mitochondrial potential, an increase in the average cell brightness is consistent with an increase in mitochondrial potential. (5) The average cellular brightness was also calculated by dividing the integrated intensity of an entire field of
20 cells stained with Bodipy FL phalloidin dye by the number of nuclei in that field. Because the phallotoxins bind with high affinity to the polymerized form of actin, the amount of Bodipy FL phalloidin dye that accumulates within the cell is proportional to actin polymerization state. An increase in the average cell brightness is consistent with an increase in actin polymerization.

25 **Results.** Figure 24 (top panels) shows the changes paclitaxel induced in the nuclear morphology of L-929 cells. Increasing amounts of paclitaxel caused nuclei to enlarge and fragment 293, a hallmark of apoptosis. Quantitative analysis of these and other images obtained by the cell screening system is presented in the same figure. Each parameter measured showed that the L-929 cells 296 were less sensitive to low
30 concentrations of paclitaxel than were SNB-19 cells 297. At higher concentrations though, the L-929 cells showed a response for each parameter measured. The multiparameter approach of this assay is useful in dissecting the mechanisms of drug

action. For example, the area, brightness, and fragmentation of the nucleus 298 and actin polymerization values 294 reached a maximum value when SNB-19 cells were treated with 10 nM paclitaxel (Figure 24; top and bottom graphs). However, mitochondrial potential 295 was minimal at the same concentration of paclitaxel (Figure 24; middle graph). The fact that all the parameters measured approached control levels at increasing paclitaxel concentrations (>10 nM) suggests that SNB-19 cells have low affinity drug metabolic or clearance pathways that are compensatory at sufficiently high levels of the drug. Contrasting the drug sensitivity of SNB-19 cells 297, L-929 showed a different response to paclitaxel 296. These fibroblastic cells showed a maximal response in many parameters at 5 μ M paclitaxel, a 500-fold higher dose than SNB-19 cells. Furthermore, the L-929 cells did not show a sharp decrease in mitochondrial potential 295 at any of the paclitaxel concentrations tested. This result is consistent with the presence of unique apoptosis pathways between a normal and cancer cell line. Therefore, these results indicate that a relatively simple fluorescence labeling protocol can be coupled with the cell screening system of the present invention to produce a high-content screen of key events involved in programmed cell death.

Background

A key to the mechanism of apoptosis was the discovery that, irrespective of the lethal stimulus, death results in identical apoptotic morphology that includes cell and organelle dismantling and repackaging, DNA cleavage to nucleosome sized fragments, and engulfment of the fragmented cell to avoid an inflammatory response. Apoptosis is therefore distinct from necrosis, which is mediated more by acute trauma to a cell, resulting in spillage of potentially toxic and antigenic cellular components into the intercellular milieu, leading to an inflammatory response.

The criteria for determining whether a cell is undergoing apoptosis (Wyllie et al. 1980. *Int Rev Cytol.* 68:251-306; Thompson, 1995. *Science.* 267:1456-62; Majno and Joris. 1995. *Am J Pathol.* 146:3-15; Allen et al. 1998. *Cell Mol Life Sci.* 54:427-45) include distinct morphological changes in the appearance of the cell, as well as alterations in biochemical and molecular markers. For example, apoptotic cells often undergo cytoplasmic membrane blebbing, their chromosomes rapidly condense and

aggregate around the nuclear periphery, the nucleus fragments, and small apoptotic bodies are formed. In many, but not all, apoptotic cells, chromatin becomes a target for specific nucleases that cleave the DNA.

Apoptosis is commonly accompanied by a characteristic change in nuclear morphology (chromatin condensation or fragmentation) and a step-wise fragmentation of DNA culminating in the formation of mono- and/or oligomeric fragments of 200 base pairs. Specific changes in organellar function, such as mitochondrial membrane potential, occur. In addition, specific cysteine proteases (caspases) are activated, which catalyzes a highly selective pattern of protein degradation by proteolytic cleavage after specific aspartic acid residues. In addition, the external surface exposure of phosphatidylserine residues (normally on the inner membrane leaflet) allows for the recognition and elimination of apoptotic cells, before the membrane breaks up and cytosol or organelles spill into the intercellular space and elicit inflammatory reactions. Moreover, cells undergoing apoptosis tend to shrink, while also having a reduced intracellular potassium level.

The general patterns of apoptotic signals are very similar among different cell types and apoptotic inducers. However, the details of the pathways actually vary significantly depending on cell type and inducer. The dependence and independence of various signal transduction pathways involved in apoptosis are currently topics of intense research. We show here that the pathway also varies depending upon the dose of the inducer in specific cell types.

Nuclear Morphology

Cells undergoing apoptosis generally exhibit two types of nuclear change, fragmentation or condensation ((Majno and Joris, 1995), (Earnshaw, 1995)). The response in a given cell type appears to vary depending on the apoptotic inducer. During nuclear fragmentation, a circular or oval nucleus becomes increasingly lobular. Eventually, the nucleus fragments dramatically into multiple sub-nuclei. Sometimes the density of the chromatin within the lobular nucleus may show spatial variations in distribution (heterochromatization), approximating the margination seen in nuclear condensation.

Nuclear condensation has been reported in some cell types, such as MCF-7 (Saunders et al. 1997. *Int J Cancer*. 70:214-20). Condensation appears to arise as a consequence of the loss of structural integrity of the euchromatin, nuclear matrix and nuclear lamina (Hendzel et al. 1998. *J Biol Chem*. 273:24470-8). During nuclear
5 condensation, the chromatin concentrates near the margin of the nucleus, leading to the overall shrinkage of the nucleus. Thus, the use of nuclear morphology as a measure of apoptosis must take both condensation and fragmentation into account.

Material and Methods

10 Cells were plated into 96-well plates at densities of 3×10^3 to 1×10^4 cells/well. The following day apoptotic inducers were added at indicated concentrations and cells were incubated for indicated time periods (usually 16-30 hours). The next day medium was removed and cells were stained with 5 $\mu\text{g/ml}$ Hoechst (Molecular Probes, Inc.) in fresh medium and incubated for 30 minutes at 37°C. Cells were washed in Hank's
15 Balanced Salt Solution (HBSS) and fixed with 3.7% formaldehyde in HBSS at room temperature. Cells were washed 2X with HBSS at room temperature and the plate was sealed.

Quantitation of changes in nuclear morphology upon induction of apoptosis was accomplished by (1) measuring the effective size of the nuclear region; and (2)
20 measuring the degree of convolution of the perimeter. The size parameter provides the more sensitive measure of nuclear condensation, whereas the perimeter measure provides a more sensitive measure of nuclear fragmentation.

Results & Discussion

25 L929 cells responded to both staurosporine (30 hours) and paclitaxel (30 hours) with a dose-dependent change in nuclear morphology (Fig 25A and 25B). BHK cells illustrated a slightly more complicated, yet clearly visible response. Staurosporine appeared to stimulate nuclear condensation at lower doses and nuclear fragmentation at higher doses (Fig 25C and 25D). In contrast, paclitaxel induced a consistent increase in
30 nuclear fragmentation with increasing concentrations. The response of MCF-7 cells varied dramatically depending upon the apoptotic inducer. Staurosporine appeared to

elicit nuclear condensation whereas paclitaxel induced nuclear fragmentation (Fig 25E and 25F).

Figure 26 illustrates the dose response of cells in terms of both nuclear size and nuclear perimeter convolution. There appears to be a swelling of the nuclei that precedes the fragmentation.

Result of evaluation: Differential responses by cell lines and by apoptotic inducers were observed in a dose dependent manner, indicating that this assay will be useful for detecting changes in the nucleus characteristic of apoptosis.

10 Actin reorganization

We assessed changes in the actin cytoskeleton as a potential parameter related to apoptotic changes. This was based on preliminary observations of an early increase in f-actin content detected with fluorescent phalloidin labeling, an f-actin specific stain (our unpublished data; Levee et al. 1996. *Am J Physiol.* 271:C1981-92; Maekawa et al. 1996. *Clin Exp Immunol.* 105:389-96). Changes in the actin cytoskeleton during apoptosis have not been observed in all cell types. (Endresen et al. 1995. *Cytometry.* 20:162-71, van Engeland et al. 1997. *Exp Cell Res.* 235:421-30).

Material and Methods

Cells were plated in 96-well plates at densities of 3×10^3 to 1×10^4 cells/well. The following day apoptotic inducers were added at indicated concentrations. Cells were incubated for the indicated time periods (usually 16-30 hours). The next day the medium was removed and cells were stained with 5 $\mu\text{g/ml}$ Hoechst (Molecular Probes, Inc.) in fresh medium and incubated for 30 minutes at 30°C. Cells were washed in HBSS and fixed with 3.7% formaldehyde in HBSS at room temperature. Plates were washed with HBSS and permeabilized with 0.5% v/v Triton X-100 in HBSS at room temperature. Plates were washed in HBSS and stained with 100 μl of 1U/ml of Alexa 488 Phalloidin stock (100 $\mu\text{l/well}$, Molecular Probes, Inc.). Cells were washed 2X with HBSS at RT and the plate was sealed.

Quantitation of f-actin content was accomplished by measuring the intensity of phalloidin staining around the nucleus. This was determined to be a reasonable approximation of a full cytoplasmic average of the intensity. The mask used to approximate this cytoplasmic measure was derived from the nuclear mask defined by

the Hoechst stain. Derivation was accomplished by combinations of erosions and dilations.

Results and Discussion

5 Changes in f-actin content varied based on cell type and apoptotic inducer (Fig 27). Staurosporine (30 hours) induced increases in f-actin in L929 (Fig. 27A) and BHK (Fig. 27B) cells. MCF-7 cells exhibited a concentration-dependent response. At low concentrations (Fig. 27E) there appeared to be a decrease in f-actin content. At higher concentrations, f-actin content increased. Paclitaxel (30 hours) treatment led to a wide
10 variety of responses. L929 cells responded with graded increases in f-actin (Fig. 27B) whereas both BHK and MCF-7 responses were highly variable (Figs. 27D & 27F, respectively).

Result of Evaluation: Both increases and decreases in signal intensity were
15 measured for several cell lines and found to exhibit a concentration dependent response. For certain cell line/apoptotic inducer pairs this could be a statistically significant apoptotic indicator.

Changes in Mitochondrial Mass/Potential

20 Introduction

 Changes in mitochondria play a central role in apoptosis (Henkart and Grinstein. 1996. *J Exp Med.* 183:1293-5). Mitochondria release apoptogenic factors through the outer membrane and dissipate the electrochemical gradient of the inner membrane. This is thought to occur via formation of the mitochondria permeability
25 transition (MPT), although it is apparently not true in all cases. An obvious manifestation of the formation of the MPT is collapse of the mitochondrial membrane potential. Inhibition of MPT by pharmacological intervention or mitochondrial expression of the anti-apoptotic protein Bcl-2 prevents cell death, suggesting the formation of the MPT may be a rate-limiting event of the death process (For review
30 see: Kroemer et al. 1998. *Annu Rev Physiol.* 60:619-42). It has also been observed that mitochondria can proliferate during stimulation of apoptosis (Mancini et al. 1997. *J Cell Biol.* 138:449-69; Camilleri-Broet et al. 1998. *Exp Cell Res.* 239:277-92).

One approach for measuring apoptosis-induced changes in mitochondria is to measure the mitochondrial membrane potential. Of the methods available, the simplest measure is the redistribution of a cationic dye that distributes within intracellular organelles based on the membrane potential. Such an approach traditionally requires live cells for the measurements. The recent introduction of the MitoTracker dyes (Poot et al. 1997. *Cytometry*. 27:358-64; available from Molecular Probes, Inc., Oregon) provides a means of measuring mitochondrial membrane potential after fixation.

Given the observations of a possible increase in mitochondrial mass during apoptosis, the amount of dye labeling the mitochondria is related to both membrane potential and the number of mitochondria. If the number of mitochondria remains constant then the amount of dye is directly related to the membrane potential. If the number of mitochondria is not constant, then the signal will likely be dominated by the increase in mass (Reipert et al. 1995. *Exp Cell Res*. 221:281-8).

Probes are available that allow a clear separation between changes in mass and potential in HCS assays. Mitochondrial mass is measured directly by labeling with Mitotracker Green FM (Poot and Pierce, 1999, *Cytometry*. 35:311-7; available from Molecular Probes, Inc., Oregon). The labeling is independent of mitochondrial membrane potential but proportional to mitochondrial mass. This also provides a means of normalizing other mitochondrial measures in each cell with respect to mitochondrial mass.

Material and Methods

Cells were plated into 96-well plates at densities of 3×10^3 to 1×10^4 cells/well. The following day apoptotic inducers were added at the indicated concentrations and cells were incubated for the indicated time periods (usually 16-30 hours). Cells were stained with 5 $\mu\text{g/ml}$ Hoechst (Molecular Probes, Inc.) and 750 nM MitoTracker Red (CMXRos, Molecular Probes, Inc.) in fresh medium and incubated for 30 minutes at 37°C. Cells were washed in HBSS and fixed with 3.7% formaldehyde in HBSS at room temperature. Plates were washed with HBSS and permeabilized with 0.5% v/v Triton X-100 in HBSS at room temperature. Cells were washed 2X with HBSS at room temperature and the plate was sealed. For dual labeling of mitochondria, cells were

treated with 200 nM Mitotracker Green and 200 nM Mitotracker Red for 0.5 hours before fixation.

Results & Discussion

5 Induction of apoptosis by staurosporine and paclitaxel led to varying mitochondrial changes depending upon the stimulus. L929 cells exhibited a clear increase in mitochondrial mass with increasing staurosporine concentrations (Fig. 28). BHK cells exhibited either a decrease in membrane potential at lower concentrations of staurosporine, or an increase in mass at higher concentrations of staurosporine (Fig. 10 28C). MCF-7 cells responded by a consistent decrease in mitochondrial membrane potential in response to increasing concentrations of staurosporine (Fig 28E). Increasing concentrations of paclitaxel caused consistent increases in mitochondrial mass (Fig 28B, 28D, and 28F).

The mitochondrial membrane potential is measured by labeling mitochondria 15 with both Mitotracker Green FM and Mitotracker Red (Molecular Probes, Inc). Mitotracker Red labeling is proportional to both mass and membrane potential. Mitotracker Green FM labeling is proportional to mass. The ratio of Mitotracker Red signal to the Mitotracker Green FM signal provides a measure of mitochondrial membrane potential (Poot and Pierce, 1999). This ratio normalizes the mitochondrial 20 mass with respect to the Mitotracker Red signal. (See Figure 28G) Combining the ability to normalize to mitochondrial mass with a measure of the membrane potential allows independent assessment of both parameters.

Result of Evaluation: Both decreases in potential and increases in mass were observed 25 depending on the cell line and inducer tested. Dose dependent correlation demonstrates that this is a promising apoptotic indicator.

It is possible to combine multiple measures of apoptosis by exploiting the spectral domain of fluorescence spectroscopy. In fact, all of the nuclear morphology/f-actin content/mitochondrial mass/mitochondrial potential data shown earlier were 30 collected as multiparameter assays, but were presented individually for clarity.

Example 7. Protease induced translocation of a signaling enzyme containing a disease-associated sequence from cytoplasm to nucleus.

Plasmid construct. A eukaryotic expression plasmid containing a coding
5 sequence for a green fluorescent protein – caspase (Cohen (1997), *Biochemical J.*
326:1-16; Liang et al. (1997), *J. of Molec. Biol.* 274:291-302) chimera is prepared using
GFP mutants. The construct is used to transfect eukaryotic cells.

Cell preparation and transfection. Cells are trypsinized and plated 24 h prior
to transfection and incubated at 37°C and 5% CO₂. Transfections are performed by
10 methods including, but not limited to calcium phosphate coprecipitation or lipofection.
Cells are incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C
and 5% CO₂, washed 3-4 times with DMEM to remove the precipitate, followed by the
addition of C-DMEM. Lipofectamine transfections are performed in serum-free
DMEM without antibiotics according to the manufacturer's instructions. Following a
15 2-3 hour incubation with the DNA-liposome complexes, the medium is removed and
replaced with C-DMEM.

Apoptotic induction of Caspase-GFP translocation. To obtain Caspase-GFP
translocation kinetic data, nuclei of transfected cells are first labeled with 5 µg/ml
Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 37°C and 5% CO₂.
20 Cells are washed once in Hank's Balanced Salt Solution (HBSS) followed by the
addition of compounds that induce apoptosis. These compounds include, but are not
limited to paclitaxel, staurosporine, ceramide, and tumor necrosis factor. To obtain
fixed time point titration data, transfected cells are first washed with DMEM and then
incubated at 37°C and 5% CO₂ for 1 h in the presence of 0 – 1000 nM compound in
25 DMEM. Cells are analyzed live or they are rinsed with HBSS, fixed for 15 min with
3.7% formaldehyde in HBSS, stained with Hoechst 33342, and washed before analysis.

Image acquisition and analysis. Kinetic data are collected by acquiring
fluorescence image pairs (Caspase-GFP and Hoechst 33342-labeled nuclei) from fields
of living cells at 1 min intervals for 30 min after the addition of compound. Likewise,
30 image pairs are obtained from each well of the fixed time point screening plates 1 h
after the addition of compound. In both cases, the image pairs obtained at each time
point are used to define nuclear and cytoplasmic regions in each cell. Translocation of

Caspase-GFP is calculated by dividing the integrated fluorescence intensity of Caspase-GFP in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. In the fixed time point screen this translocation ratio is calculated from data obtained from at least 200 cells at each concentration of compound tested. Drug-induced translocation of Caspase-GFP from the cytoplasm to the nucleus is therefore correlated with an increase in the translocation ratio. Molecular interaction libraries including, but not limited to those comprising putative activators or inhibitors of apoptosis-activated enzymes are used to screen the indicator cell lines and identify a specific ligand for the DAS, and a pathway activated by compound activity.

Example 8. Identification of novel steroid receptors from DAS

Two sources of material and/or information are required to make use of this embodiment, which allows assessment of the function of an uncharacterized gene. First, disease associated sequence bank(s) containing cDNA sequences suitable for transfection into mammalian cells can be used. Because every RADE or differential expression experiment generates up to several hundred sequences, it is possible to generate an ample supply of DAS. Second, information from primary sequence database searches can be used to place DAS into broad categories, including, but not limited to, those that contain signal sequences, seven trans-membrane motifs, conserved protease active site domains, or other identifiable motifs. Based on the information acquired from these sources, method types and indicator cell lines to be transfected are selected. A large number of motifs are already well characterized and encoded in the linear sequences contained within the large number genes in existing genomic databases.

In one embodiment, the following steps are taken:

1) Information from the DAS identification experiment (including database searches) is used as the basis for selecting the relevant biological processes. (for example, look at the DAS from a tumor line for cell cycle modulation, apoptosis, metastatic proteases, etc.)

2) Sorting of DNA sequences or DAS by identifiable motifs (ie. signal sequences, 7- transmembrane domains, conserved protease active site domains, etc.) This initial grouping will determine fluorescent tagging strategies, host cell lines,

indicator cell lines, and banks of bioactive molecules to be screened, as described *supra*.

- 5 3) Using well established molecular biology methods, ligate DAS into an expression vector designed for this purpose. Generalized expression vectors contain promoters, enhancers, and terminators for which to deliver target sequences to the cell for transient expression. Such vectors may also contain antibody tagging sequences, direct association sequences, chromophore fusion sequences like GFP, etc. to facilitate detection when expressed by the host.

- 10 4) Transiently transfect cells with DAS containing vectors using standard transfection protocols including: calcium phosphate co-precipitation, liposome mediated, DEAE dextran mediated, polycationic mediated, viral mediated, or electroporation, and plate into microtiter plates or microwell arrays. Alternatively, transfection can be done directly in the microtiter plate itself.

- 15 5) Carry out the cell screening methods as described *supra*.

In this embodiment, DAS shown to possess a motif(s) suggestive of transcriptional activation potential (for example, DNA binding domain, amino terminal modulating domain, hinge region, or carboxy terminal ligand binding domain) are utilized to identify novel steroid receptors.

- 20 Defining the fluorescent tags for this experiment involves identification of the nucleus through staining, and tagging the DAS by creating a GFP chimera via insertion of DAS into an expression vector, proximally fused to the gene encoding GFP. Alternatively, a single chain antibody fragment with high affinity to some portion of the expressed DAS could be constructed using technology available in the art (Cambridge
25 Antibody Technologies) and linked to a fluorophore (FITC) to tag the putative transcriptional activator/receptor in the cells. This alternative would provide an external tag requiring no DNA transfection and therefore would be useful if distribution data were to be gathered from the original primary cultures used to generate the DAS.

- Plasmid construct.** A eukaryotic expression plasmid containing a coding
30 sequence for a green fluorescent protein – DAS chimera is prepared using GFP mutants. The construct is used to transfect HeLa cells. The plasmid, when transfected into the host cell, produces a GFP fused to the DAS protein product, designated GFP-DASpp.

Cell preparation and transfection. HeLa cells are trypsinized and plated using DMEM containing 5% charcoal/dextran-treated fetal bovine serum (FBS) (Hyclone) and 1% penicillin-streptomycin (C-DMEM) 12-24 hours prior to transfection and incubated at 37°C and 5% CO₂. Transfections are performed by calcium phosphate coprecipitation or with Lipofectamine (Life Technologies). For the calcium phosphate transfections, the medium is replaced, prior to transfection, with DMEM containing 5% charcoal/dextran-treated FBS. Cells are incubated with the calcium phosphate-DNA precipitate for 4-5 hours at 37°C and 5% CO₂, and washed 3-4 times with DMEM to remove the precipitate, followed by the addition of C-DMEM. Lipofectamine transfections are performed in serum-free DMEM without antibiotics according to the manufacturer's instructions. Following a 2-3 hour incubation with the DNA-liposome complexes, the medium is removed and replaced with C-DMEM. All transfected cells in 96-well microtiter plates are incubated at 33°C and 5% CO₂ for 24-48 hours prior to drug treatment. Experiments are performed with the receptor expressed transiently in HeLa cells.

Localization of expressed GFP-DASpp inside cells. To obtain cellular distribution data, nuclei of transfected cells are first labeled with 5 µg/ml Hoechst 33342 (Molecular Probes) in C-DMEM for 20 minutes at 33°C and 5% CO₂. Cells are washed once in Hank's Balanced Salt Solution (HBSS). The cells are analyzed live or they are rinsed with HBSS, fixed for 15 min with 3.7% formaldehyde in HBSS, stained with Hoechst 33342, and washed before analysis.

In a preferred embodiment, image acquisition and analysis are performed using the cell screening system of the present invention. The intracellular GFP-DASpp fluorescence signal is collected by acquiring fluorescence image pairs (GFP-DASpp and Hoechst 33342-labeled nuclei) from field cells. The image pairs obtained at each time point are used to define nuclear and cytoplasmic regions in each cell. Data demonstrating dispersed signal in the cytoplasm would be consistent with known steroid receptors that are DNA transcriptional activators.

Screening for induction of GFP-DASpp translocation. Using the above construct, confirmed for appropriate expression of the GFP-DASpp, as an indicator cell line, a screen of various ligands is performed using a series of steroid type ligands including, but not limited to: estrogen, progesterone, retinoids, growth factors,

androgens, and many other steroid and steroid based molecules. Image acquisition and analysis are performed using the cell screening system of the invention. The intracellular GFP-DASpp fluorescence signal is collected by acquiring fluorescence image pairs (GFP-DASpp and Hoechst 33342-labeled nuclei) from fields cells. The image pairs obtained at each time point are used to define nuclear and cytoplasmic regions in each cell. Translocation of GFP-DASpp is calculated by dividing the integrated fluorescence intensity of GFP-DASpp in the nucleus by the integrated fluorescence intensity of the chimera in the cytoplasm or as a nuclear-cytoplasmic difference of GFP fluorescence. A translocation from the cytoplasm into the nucleus indicates a ligand binding activation of the DASpp thus identifying the potential receptor class and action. Combining this data with other data obtained in a similar fashion using known inhibitors and modifiers of steroid receptors, would either validate the DASpp as a target, or more data would be generated from various sources.

Example 9 Additional Screens

Translocation between the plasma membrane and the cytoplasm:

Profilactin complex dissociation and binding of profilin to the plasma membrane. In one embodiment, a fluorescent protein biosensor of profilin membrane binding is prepared by labeling purified profilin (Federov et al.(1994), *J. Molec. Biol.* 241:480-482; Lanbrechts et al. (1995), *Eur. J. Biochem.* 230:281-286) with a probe possessing a fluorescence lifetime in the range of 2-300 ns. The labeled profilin is introduced into living indicator cells using bulk loading methodology and the indicator cells are treated with test compounds. Fluorescence anisotropy imaging microscopy (Gough and Taylor (1993), *J. Cell Biol.* 121:1095-1107) is used to measure test-compound dependent movement of the fluorescent derivative of profilin between the cytoplasm and membrane for a period of time after treatment ranging from 0.1 s to 10 h.

Rho-RhoGDI complex translocation to the membrane. In another embodiment, indicator cells are treated with test compounds and then fixed, washed, and permeabilized. The indicator cell plasma membrane, cytoplasm, and nucleus are all labeled with distinctly colored markers followed by immunolocalization of Rho protein (Self et al. (1995), *Methods in Enzymology* 256:3-10; Tanaka et al. (1995),

Methods in Enzymology 256:41-49) with antibodies labeled with a fourth color. Each of the four labels is imaged separately using the cell screening system, and the images used to calculate the amount of inhibition or activation of translocation effected by the test compound. To do this calculation, the images of the probes used to mark the plasma membrane and cytoplasm are used to mask the image of the immunological probe marking the location of intracellular Rho protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the plasma membrane integrated brightness/area by the cytoplasmic integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound.

β -Arrestin translocation to the plasma membrane upon G-protein receptor activation.

In another embodiment of a cytoplasm to membrane translocation high-content screen, the translocation of β -arrestin protein from the cytoplasm to the plasma membrane is measured in response to cell treatment. To measure the translocation, living indicator cells containing luminescent domain markers are treated with test compounds and the movement of the β -arrestin marker is measured in time and space using the cell screening system of the present invention. In a preferred embodiment, the indicator cells contain luminescent markers consisting of a green fluorescent protein β -arrestin (GFP- β -arrestin) protein chimera (Barak et al. (1997), *J. Biol. Chem.* 272:27497-27500; Daaka et al. (1998), *J. Biol. Chem.* 273:685-688) that is expressed by the indicator cells through the use of transient or stable cell transfection and other reporters used to mark cytoplasmic and membrane domains. When the indicator cells are in the resting state, the domain marker molecules partition predominately in the plasma membrane or in the cytoplasm. In the high-content screen, these markers are used to delineate the cell cytoplasm and plasma membrane in distinct channels of fluorescence. When the indicator cells are treated with a test compound, the dynamic redistribution of the GFP- β -arrestin is recorded as a series of images over a time scale ranging from 0.1 s to 10 h. In a preferred embodiment, the time scale is 1 h. Each image is analyzed by a method that quantifies the movement of the GFP- β -arrestin

protein chimera between the plasma membrane and the cytoplasm. To do this calculation, the images of the probes used to mark the plasma membrane and cytoplasm are used to mask the image of the GFP- β -arrestin probe marking the location of intracellular GFP- β -arrestin protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the plasma membrane integrated brightness/area by the cytoplasmic integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compounds of interest.

Translocation between the endoplasmic reticulum and the Golgi:

In one embodiment of an endoplasmic reticulum to Golgi translocation high-content screen, the translocation of a VSVG protein from the ts045 mutant strain of vesicular stomatitis virus (Ellenberg et al. (1997), *J. Cell Biol.* 138:1193-1206; Presley et al. (1997) *Nature* 389:81-85) from the endoplasmic reticulum to the Golgi domain is measured in response to cell treatment. To measure the translocation, indicator cells containing luminescent reporters are treated with test compounds and the movement of the reporters is measured in space and time using the cell screening system of the present invention. The indicator cells contain luminescent reporters consisting of a GFP-VSVG protein chimera that is expressed by the indicator cell through the use of transient or stable cell transfection and other domain markers used to measure the localization of the endoplasmic reticulum and Golgi domains. When the indicator cells are in their resting state at 40°C, the GFP-VSVG protein chimera molecules are partitioned predominately in the endoplasmic reticulum. In this high-content screen, domain markers of distinct colors used to delineate the endoplasmic reticulum and the Golgi domains in distinct channels of fluorescence. When the indicator cells are treated with a test compound and the temperature is simultaneously lowered to 32°C, the dynamic redistribution of the GFP-VSVG protein chimera is recorded as a series of images over a time scale ranging from 0.1 s to 10-h. Each image is analyzed by a method that quantifies the movement of the GFP-VSVG protein chimera between the endoplasmic reticulum and the Golgi domains. To do this calculation, the images of

the probes used to mark the endoplasmic reticulum and the Golgi domains are used to mask the image of the GFP-VSVG probe marking the location of intracellular GFP-VSVG protein. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the endoplasmic reticulum integrated brightness/area by the Golgi integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compounds of interest at final concentrations ranging from 10^{-12} M to 10^{-3} M for a period ranging from 1 min to 10 h.

Induction and inhibition of organellar function:

Intracellular microtubule stability.

In another aspect of the invention, an automated method for identifying compounds that modify microtubule structure is provided. In this embodiment, indicator cells are treated with test compounds and the distribution of luminescent microtubule-labeling molecules is measured in space and time using a cell screening system, such as the one disclosed above. The luminescent microtubule-labeling molecules may be expressed by or added to the cells either before, together with, or after contacting the cells with a test compound.

In one embodiment of this aspect of the invention, living cells express a luminescently labeled protein biosensor of microtubule dynamics, comprising a protein that labels microtubules fused to a luminescent protein. Appropriate microtubule-labeling proteins for this aspect of the invention include, but are not limited to α and β tubulin isoforms, and MAP4. Preferred embodiments of the luminescent protein include, but are not limited to green fluorescent protein (GFP) and GFP mutants. In a preferred embodiment, the method involves transfecting cells with a microtubule labeling luminescent protein, wherein the microtubule labeling protein can be, but is not limited to, α -tubulin, β -tubulin, or microtubule-associated protein 4 (MAP4). The approach outlined here enables those skilled in the art to make live cell measurements

In a most preferred embodiment, MAP4 is fused to a modified version of the *Aequorea victoria* green fluorescent protein (GFP). A DNA construct has been made which consists of a fusion between the EGFP coding sequence (available from Clontech) and the coding sequence for mouse MAP4. (Olson et al., (1995), J. Cell Biol. 130(3): 639-650). MAP4 is a ubiquitous microtubule-associated protein that is known to interact with microtubules in interphase as well as mitotic cells (Olmsted and Murofushi, (1993), MAP4. In "Guidebook to the Cytoskeleton and Motor Proteins." Oxford University Press. T. Kreis and R. Vale, eds.) Its localization, then, can serve as an indicator of the localization, organization, and integrity of microtubules in living (or fixed) cells at all stages of the cell cycle for cell-based HCS assays. While MAP2 and tau (microtubule associated proteins expressed specifically in neuronal cells) have been used to form GFP chimeras (Kaeck *et al.*, (1996) Neuron. 17: 1189-1199; Hall *et al.*, (1997), Proc. Nat. Acad. Sci. 94: 4733-4738) their restricted cell type distribution and the tendency of these proteins to bundle microtubules when overexpressed make these proteins less desirable as molecular reagents for analysis in live cells originating from varied tissues and organs. Moderate overexpression of GFP-MAP4 does not disrupt microtubule function or integrity (Olson et al., 1995). Similar constructs can be made using β -tubulin or α -tubulin via standard techniques in the art. These chimeras will provide a means to observe and analyze microtubule activity in living cells during all stages of the cell cycle.

In another embodiment, the luminescently labeled protein biosensor of microtubule dynamics is expressed, isolated, and added to the cells to be analyzed via bulk loading techniques, such as microinjection, scrape loading, and impact-mediated loading. In this embodiment, there is not an issue of overexpression within the cell, and thus α and β tubulin isoforms, MAP4, MAP2 and/or tau can all be used.

In a further embodiment, the protein biosensor is expressed by the cell, and the cell is subsequently contacted with a luminescent label, such as a labeled antibody, that detects the protein biosensor, endogenous levels of a protein antigen, or both. In this embodiment, a luminescent label that detects α and β tubulin isoforms, MAP4, MAP2 and/or tau, can be used.

A variety of GFP mutants are available, all of which would be effective in this invention, including, but not limited to, GFP mutants which are commercially available (Clontech, California).

The MAP4 construct has been introduced into several mammalian cell lines (BHK-21, Swiss 3T3, HeLa, HEK 293, LLCPK) and the organization and localization of tubulin has been visualized in live cells by virtue of the GFP fluorescence as an indicator of MAP4 localization. The construct can be expressed transiently or stable cell lines can be prepared by standard methods. Stable HeLa cell lines expressing the EGFP-MAP4 chimera have been obtained, indicating that expression of the chimera is not toxic and does not interfere with mitosis.

Possible selectable markers for establishment and maintenance of stable cell lines include, but are not limited to the neomycin resistance gene, hygromycin resistance gene, zeocin resistance gene, puromycin resistance gene, bleomycin resistance gene, and blastacidin resistance gene.

The utility of this method for the monitoring of microtubule assembly, disassembly, and rearrangement has been demonstrated by treatment of transiently and stably transfected cells with microtubule drugs such as paclitaxel, nocodazole, vincristine, or vinblastine.

The present method provides high-content and combined high throughput-high content cell-based screens for anti-microtubule drugs, particularly as one parameter in a multi-parametric cancer target screen. The EGFP-MAP4 construct used herein can also be used as one of the components of a high-content screen that measures multiple signaling pathways or physiological events. In a preferred embodiment, a combined high throughput and high content screen is employed, wherein multiple cells in each of the locations containing cells are analyzed in a high throughput mode, and only a subset of the locations containing cells are analyzed in a high content mode. The high throughput screen can be any screen that would be useful to identify those locations containing cells that should be further analyzed, including, but not limited to, identifying locations with increased luminescence intensity, those exhibiting expression of a reporter gene, those undergoing calcium changes, and those undergoing pH changes.

In addition to drug screening applications, the present invention may be applied to clinical diagnostics, the detection of chemical and biological warfare weapons, and the basic research market since fundamental cell processes, such as cell division and motility, are highly dependent upon microtubule dynamics.

5

Image Acquisition and Analysis

Image data can be obtained from either fixed or living indicator cells. To extract morphometric data from each of the images obtained the following method of analysis is used:

- 10 1. Threshold each nucleus and cytoplasmic image to produce a mask that has value = 0 for each pixel outside a nucleus or cell boundary.
2. Overlay the mask on the original image, detect each object in the field (*i.e.*, nucleus or cell), and calculate its size, shape, and integrated intensity.
3. Overlay the whole cell mask obtained above on the corresponding luminescent
15 microtubule image and apply one or more of the following set of classifiers to determine the microtubule morphology and the effect of drugs on microtubule morphology.

Microtubule morphology is defined using a set of classifiers to quantify aspects of microtubule shape, size, aggregation state, and polymerization state. These
20 classifiers can be based on approaches that include co-occurrence matrices, texture measurements, spectral methods, structural methods, wavelet transforms, statistical methods, or combinations thereof. Examples of such classifiers are as follows:

1. A classifier to quantify microtubule length and width using edge
25 detection methods such as that discussed in Kolega et al. ((1993). *BioImaging* 1:136-150), which discloses a non-automated method to determine edge strength in individual cells), to calculate the total edge strength within each cell. To normalize for cell size, the total edge strength can be divided by the cell area to give a "microtubule morphology" value. Large microtubule morphology values are associated with strong edge strength values and are therefore maximal in cells containing distinct microtubule
30 structures. Likewise, small microtubule morphology values are associated with weak edge strength and are minimal in cells with depolymerized microtubules. The physiological range of microtubule morphology values is set by treating cells with either the microtubule stabilizing drug paclitaxel (10 μ M) or the microtubule depolymerizing drug nocodazole (10 μ g/ml).

35

2. A classifier to quantify microtubule aggregation into punctate spots or foci using methodology from the receptor internalization methods discussed supra.

3. A classifier to quantify microtubule depolymerization using a measure of image texture.

5 4. A classifier to quantify apparent interconnectivity, or branching (or both), of the microtubules.

5. Measurement of the kinetics of microtubule reorganization using the above classifiers on a time series of images of cells treated with test compounds.

10 In a further aspect, kits are provided for analyzing microtubule stability, comprising an expression vector comprising a nucleic acid that encodes a microtubule labeling protein and instructions for using the expression vector for carrying out the methods described above. In a preferred embodiment, the expression vector further
15 comprises a nucleic acid that encodes a luminescent protein, wherein the microtubule binding protein and the luminescent protein thereof are expressed as a fusion protein. Alternatively, the kit may contain an antibody that specifically binds to the microtubule-labeling protein. In a further embodiment, the kit includes cells that express the microtubule labeling protein. In a preferred embodiment, the cells are
20 transfected with the expression vector. In another preferred embodiment, the kits further contain a compound that is known to disrupt microtubule structure, including but not limited to curacin, nocodazole, vincristine, or vinblastine. In another preferred embodiment, the kits further comprise a compound that is known to stabilize microtubule structure, including but not limited to taxol (paclitaxel), and
25 discodermolide.

In another aspect, the present invention comprises a machine readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute the disclosed methods for analyzing microtubule stability, wherein the cell screening system comprises an optical system with a stage adapted for
30 holding a plate containing cells, a digital camera, a means for directing fluorescence or luminescence emitted from the cells to the digital camera, and a computer means for receiving and processing the digital data from the digital camera.

High-content screens involving the functional localization of macromolecules

Within this class of high-content screen, the functional localization of macromolecules in response to external stimuli is measured within living cells.

Glycolytic enzyme activity regulation. In a preferred embodiment of a cellular enzyme activity high-content screen, the activity of key glycolytic regulatory enzymes are measured in treated cells. To measure enzyme activity, indicator cells containing luminescent labeling reagents are treated with test compounds and the activity of the reporters is measured in space and time using cell screening system of the present invention.

In one embodiment, the reporter of intracellular enzyme activity is fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase (PFK-2), a regulatory enzyme whose phosphorylation state indicates intracellular carbohydrate anabolism or catabolism (Deprez et al. (1997) *J. Biol. Chem.* 272:17269-17275; Kealer et al. (1996) *FEBS Letters* 395:225-227; Lee et al. (1996), *Biochemistry* 35:6010-6019). The indicator cells contain luminescent reporters consisting of a fluorescent protein biosensor of PFK-2 phosphorylation. The fluorescent protein biosensor is constructed by introducing an environmentally sensitive fluorescent dye near to the known phosphorylation site of the enzyme (Deprez et al. (1997), *supra*; Giuliano et al. (1995), *supra*). The dye can be of the ketocyanine class (Kessler and Wolfbeis (1991), *Spectrochimica Acta* 47A:187-192) or any class that contains a protein reactive moiety and a fluorochrome whose excitation or emission spectrum is sensitive to solution polarity. The fluorescent protein biosensor is introduced into the indicator cells using bulk loading methodology.

Living indicator cells are treated with test compounds, at final concentrations ranging from 10^{-12} M to 10^{-3} M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells by collecting a spectral pair of fluorescence images at each time point. To extract morphometric data from each time point, a ratio is made between each pair of images by numerically dividing the two spectral images at each time point, pixel by pixel. Each pixel value is then used to calculate the fractional phosphorylation of PFK-2. At small fractional values of phosphorylation, PFK-2 stimulates carbohydrate catabolism.

At high fractional values of phosphorylation, PFK-2 stimulates carbohydrate anabolism.

Protein kinase A activity and localization of subunits. In another embodiment of a high-content screen, both the domain localization and activity of protein kinase A (PKA) within indicator cells are measured in response to treatment with test compounds.

The indicator cells contain luminescent reporters including a fluorescent protein biosensor of PKA activation. The fluorescent protein biosensor is constructed by introducing an environmentally sensitive fluorescent dye into the catalytic subunit of PKA near the site known to interact with the regulatory subunit of PKA (Harootunian et al. (1993), *Mol. Biol. of the Cell* 4:993-1002; Johnson et al. (1996), *Cell* 85:149-158; Giuliano et al. (1995), *supra*). The dye can be of the ketocyanine class (Kessler, and Wolfbeis (1991), *Spectrochimica Acta* 47A:187-192) or any class that contains a protein reactive moiety and a fluorochrome whose excitation or emission spectrum is sensitive to solution polarity. The fluorescent protein biosensor of PKA activation is introduced into the indicator cells using bulk loading methodology.

In one embodiment, living indicator cells are treated with test compounds, at final concentrations ranging from 10^{-12} M to 10^{-3} M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells. To extract biosensor data from each time point, a ratio is made between each pair of images, and each pixel value is then used to calculate the fractional activation of PKA (e.g., separation of the catalytic and regulatory subunits after cAMP binding). At high fractional values of activity, PFK-2 stimulates biochemical cascades within the living cell.

To measure the translocation of the catalytic subunit of PKA, indicator cells containing luminescent reporters are treated with test compounds and the movement of the reporters is measured in space and time using the cell screening system. The indicator cells contain luminescent reporters consisting of domain markers used to measure the localization of the cytoplasmic and nuclear domains. When the indicator cells are treated with a test compounds, the dynamic redistribution of a PKA fluorescent protein biosensor is recorded intracellularly as a series of images over a

time scale ranging from 0.1 s to 10 h. Each image is analyzed by a method that quantifies the movement of the PKA between the cytoplasmic and nuclear domains. To do this calculation, the images of the probes used to mark the cytoplasmic and nuclear domains are used to mask the image of the PKA fluorescent protein biosensor. The integrated brightness per unit area under each mask is used to form a translocation quotient by dividing the cytoplasmic integrated brightness/area by the nuclear integrated brightness/area. By comparing the translocation quotient values from control and experimental wells, the percent translocation is calculated for each potential lead compound. The output of the high-content screen relates quantitative data describing the magnitude of the translocation within a large number of individual cells that have been treated with test compound in the concentration range of 10^{-12} M to 10^{-3} M.

High-content screens involving the induction or inhibition of gene expression

RNA-based fluorescent biosensors

Cytoskeletal protein transcription and message localization. Regulation of the general classes of cell physiological responses including cell-substrate adhesion, cell-cell adhesion, signal transduction, cell-cycle events, intermediary and signaling molecule metabolism, cell locomotion, cell-cell communication, and cell death can involve the alteration of gene expression. High-content screens can also be designed to measure this class of physiological response.

In one embodiment, the reporter of intracellular gene expression is an oligonucleotide that can hybridize with the target mRNA and alter its fluorescence signal. In a preferred embodiment, the oligonucleotide is a molecular beacon (Tyagi and Kramer (1996) *Nat. Biotechnol.* 14:303-308), a luminescence-based reagent whose fluorescence signal is dependent on intermolecular and intramolecular interactions. The fluorescent biosensor is constructed by introducing a fluorescence energy transfer pair of fluorescent dyes such that there is one at each end (5' and 3') of the reagent. The dyes can be of any class that contains a protein reactive moiety and fluorochromes whose excitation and emission spectra overlap sufficiently to provide fluorescence energy transfer between the dyes in the resting state, including, but not limited to, fluorescein and rhodamine (Molecular Probes, Inc.). In a preferred embodiment, a

portion of the message coding for β -actin (Kislauskis et al. (1994), *J. Cell Biol.* 127:441-451; McCann et al. (1997), *Proc. Natl. Acad. Sci.* 94:5679-5684; Sutoh (1982), *Biochemistry* 21:3654-3661) is inserted into the loop region of a hairpin-shaped oligonucleotide with the ends tethered together due to intramolecular hybridization. At each end of the biosensor a fluorescence donor (fluorescein) and a fluorescence acceptor (rhodamine) are covalently bound. In the tethered state, the fluorescence energy transfer is maximal and therefore indicative of an unhybridized molecule. When hybridized with the mRNA coding for β -actin, the tether is broken and energy transfer is lost. The complete fluorescent biosensor is introduced into the indicator cells using bulk loading methodology.

In one embodiment, living indicator cells are treated with test compounds, at final concentrations ranging from 10^{-12} M to 10^{-3} M for times ranging from 0.1 s to 10 h. In a preferred embodiment, ratio image data are obtained from living treated indicator cells. To extract morphometric data from each time point, a ratio is made between each pair of images, and each pixel value is then used to calculate the fractional hybridization of the labeled nucleotide. At small fractional values of hybridization little expression of β -actin is indicated. At high fractional values of hybridization, maximal expression of β -actin is indicated. Furthermore, the distribution of hybridized molecules within the cytoplasm of the indicator cells is also a measure of the physiological response of the indicator cells.

Cell surface binding of a ligand

Labeled insulin binding to its cell surface receptor in living cells. Cells whose plasma membrane domain has been labeled with a labeling reagent of a particular color are incubated with a solution containing insulin molecules (Lee et al. (1997), *Biochemistry* 36:2701-2708; Martinez-Zaguilan et al. (1996), *Am. J. Physiol.* 270:C1438-C1446) that are labeled with a luminescent probe of a different color for an appropriate time under the appropriate conditions. After incubation, unbound insulin molecules are washed away, the cells fixed and the distribution and concentration of the insulin on the plasma membrane is measured. To do this, the cell membrane image is used as a mask for the insulin image. The integrated intensity from the masked insulin image is compared to a set of images containing known amounts of labeled insulin.

The amount of insulin bound to the cell is determined from the standards and used in conjunction with the total concentration of insulin incubated with the cell to calculate a dissociation constant or insulin to its cell surface receptor.

5 *Labeling of cellular compartments*

Whole cell labeling

Whole cell labeling is accomplished by labeling cellular components such that dynamics of cell shape and motility of the cell can be measured over time by analyzing fluorescence images of cells.

10 In one embodiment, small reactive fluorescent molecules are introduced into living cells. These membrane-permeant molecules both diffuse through and react with protein components in the plasma membrane. Dye molecules react with intracellular molecules to both increase the fluorescence signal emitted from each molecule and to entrap the fluorescent dye within living cells. These molecules include reactive
15 chloromethyl derivatives of aminocoumarins, hydroxycoumarins, eosin diacetate, fluorescein diacetate, some Bodipy dye derivatives, and tetramethylrhodamine. The reactivity of these dyes toward macromolecules includes free primary amino groups and free sulfhydryl groups.

In another embodiment, the cell surface is labeled by allowing the cell to
20 interact with fluorescently labeled antibodies or lectins (Sigma Chemical Company, St. Louis, MO) that react specifically with molecules on the cell surface. Cell surface protein chimeras expressed by the cell of interest that contain a green fluorescent protein, or mutant thereof, component can also be used to fluorescently label the entire cell surface. Once the entire cell is labeled, images of the entire cell or cell array can
25 become a parameter in high content screens, involving the measurement of cell shape, motility, size, and growth and division.

Plasma membrane labeling

In one embodiment, labeling the whole plasma membrane employs some of the
30 same methodology described above for labeling the entire cells. Luminescent molecules that label the entire cell surface act to delineate the plasma membrane.

In a second embodiment subdomains of the plasma membrane, the extracellular surface, the lipid bilayer, and the intracellular surface can be labeled separately and used as components of high content screens. In the first embodiment, the extracellular surface is labeled using a brief treatment with a reactive fluorescent molecule such as the succinimidyl ester or iodoacetamide derivatives of fluorescent dyes such as the fluoresceins, rhodamines, cyanines, and Bodipys.

In a third embodiment, the extracellular surface is labeled using fluorescently labeled macromolecules with a high affinity for cell surface molecules. These include fluorescently labeled lectins such as the fluorescein, rhodamine, and cyanine derivatives of lectins derived from jack bean (Con A), red kidney bean (erythroagglutinin PHA-E), or wheat germ.

In a fourth embodiment, fluorescently labeled antibodies with a high affinity for cell surface components are used to label the extracellular region of the plasma membrane. Extracellular regions of cell surface receptors and ion channels are examples of proteins that can be labeled with antibodies.

In a fifth embodiment, the lipid bilayer of the plasma membrane is labeled with fluorescent molecules. These molecules include fluorescent dyes attached to long chain hydrophobic molecules that interact strongly with the hydrophobic region in the center of the plasma membrane lipid bilayer. Examples of these dyes include the PKH series of dyes (U.S. 4,783,401, 4,762,701, and 4,859,584; available commercially from Sigma Chemical Company, St. Louis, MO), fluorescent phospholipids such as nitrobenzoxadiazole glycerophosphoethanolamine and fluorescein-derivatized dihexadecanoylglycerophosphoethanolamine, fluorescent fatty acids such as 5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-nonanoic acid and 1-pyrenedecanoic acid (Molecular Probes, Inc.), fluorescent sterols including cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate and cholesteryl 1-pyrenehexanoate, and fluorescently labeled proteins that interact specifically with lipid bilayer components such as the fluorescein derivative of annexin V (Caltag Antibody Co, Burlingame, CA).

In another embodiment, the intracellular component of the plasma membrane is labeled with fluorescent molecules. Examples of these molecules are the intracellular components of the trimeric G-protein receptor, adenylyl cyclase, and ionic transport

proteins. These molecules can be labeled as a result of tight binding to a fluorescently labeled specific antibody or by the incorporation of a fluorescent protein chimera that is comprised of a membrane-associated protein and the green fluorescent protein, and mutants thereof.

5

Endosome fluorescence labeling

In one embodiment, ligands that are transported into cells by receptor-mediated endocytosis are used to trace the dynamics of endosomal organelles. Examples of labeled ligands include Bodipy FL-labeled low density lipoprotein complexes, tetramethylrhodamine transferrin analogs, and fluorescently labeled epidermal growth factor (Molecular Probes, Inc.)

In a second embodiment, fluorescently labeled primary or secondary antibodies (Sigma Chemical Co. St. Louis, MO; Molecular Probes, Inc. Eugene, OR; Caltag Antibody Co.) that specifically label endosomal ligands are used to mark the endosomal compartment in cells.

In a third embodiment, endosomes are fluorescently labeled in cells expressing protein chimeras formed by fusing a green fluorescent protein, or mutants thereof, with a receptor whose internalization labels endosomes. Chimeras of the EGF, transferrin, and low density lipoprotein receptors are examples of these molecules.

20

Lysosome labeling

In one embodiment, membrane permeant lysosome-specific luminescent reagents are used to label the lysosomal compartment of living and fixed cells. These reagents include the luminescent molecules neutral red, N-(3-((2,4-dinitrophenyl)amino)propyl)-N-(3-aminopropyl)methylamine, and the LysoTracker probes which report intralysosomal pH as well as the dynamic distribution of lysosomes (Molecular Probes, Inc.)

In a second embodiment, antibodies against lysosomal antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label lysosomal components that are localized in specific lysosomal domains. Examples of these components are the degradative enzymes involved in cholesterol ester hydrolysis,

membrane protein proteases, and nucleases as well as the ATP-driven lysosomal proton pump.

In a third embodiment, protein chimeras consisting of a lysosomal protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the lysosomal domain. Examples of these components are the degradative enzymes involved in cholesterol ester hydrolysis, membrane protein proteases, and nucleases as well as the ATP-driven lysosomal proton pump.

Cytoplasmic fluorescence labeling

In one embodiment, cell permeant fluorescent dyes (Molecular Probes, Inc.) with a reactive group are reacted with living cells. Reactive dyes including monobromobimane, 5-chloromethylfluorescein diacetate, carboxy fluorescein diacetate succinimidyl ester, and chloromethyl tetramethylrhodamine are examples of cell permeant fluorescent dyes that are used for long term labeling of the cytoplasm of cells.

In a second embodiment, polar tracer molecules such as Lucifer yellow and cascade blue-based fluorescent dyes (Molecular Probes, Inc.) are introduced into cells using bulk loading methods and are also used for cytoplasmic labeling.

In a third embodiment, antibodies against cytoplasmic components (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to fluorescently label the cytoplasm. Examples of cytoplasmic antigens are many of the enzymes involved in intermediary metabolism. Enolase, phosphofructokinase, and acetyl-CoA dehydrogenase are examples of uniformly distributed cytoplasmic antigens.

In a fourth embodiment, protein chimeras consisting of a cytoplasmic protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the cytoplasm. Fluorescent chimeras of uniformly distributed proteins are used to label the entire cytoplasmic domain. Examples of these proteins are many of the proteins involved in intermediary metabolism and include enolase, lactate dehydrogenase, and hexokinase.

In a fifth embodiment, antibodies against cytoplasmic antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label cytoplasmic components that are localized in specific cytoplasmic sub-domains.

Examples of these components are the cytoskeletal proteins actin, tubulin, and cytokeratin. A population of these proteins within cells is assembled into discrete structures, which in this case, are fibrous. Fluorescence labeling of these proteins with antibody-based reagents therefore labels a specific sub-domain of the cytoplasm.

5 In a sixth embodiment, non-antibody-based fluorescently labeled molecules that interact strongly with cytoplasmic proteins are used to label specific cytoplasmic components. One example is a fluorescent analog of the enzyme DNase I (Molecular Probes, Inc.) Fluorescent analogs of this enzyme bind tightly and specifically to cytoplasmic actin, thus labeling a sub-domain of the cytoplasm. In another example,
10 fluorescent analogs of the mushroom toxin phalloidin or the drug paclitaxel (Molecular Probes, Inc.) are used to label components of the actin- and microtubule-cytoskeletons, respectively.

In a seventh embodiment, protein chimeras consisting of a cytoplasmic protein genetically fused to an intrinsically luminescent protein such as the green fluorescent
15 protein, or mutants thereof, are used to label specific domains of the cytoplasm. Fluorescent chimeras of highly localized proteins are used to label cytoplasmic sub-domains. Examples of these proteins are many of the proteins involved in regulating the cytoskeleton. They include the structural proteins actin, tubulin, and cytokeratin as well as the regulatory proteins microtubule associated protein 4 and α -actinin.

20 Nuclear labeling

In one embodiment, membrane permeant nucleic-acid-specific luminescent reagents (Molecular Probes, Inc.) are used to label the nucleus of living and fixed cells. These reagents include cyanine-based dyes (*e.g.*, TOTO[®], YOYO[®], and BOBO[™]),
25 phenanthridines and acridines (*e.g.*, ethidium bromide, propidium iodide, and acridine orange), indoles and imidazoles (*e.g.*, Hoechst 33258, Hoechst 33342, and 4',6-diamidino-2-phenylindole), and other similar reagents (*e.g.*, 7-aminoactinomycin D, hydroxystilbamidine, and the psoralens).

In a second embodiment, antibodies against nuclear antigens (Sigma Chemical
30 Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label nuclear components that are localized in specific nuclear domains. Examples of these components are the macromolecules involved in maintaining DNA structure and

function. DNA, RNA, histones, DNA polymerase, RNA polymerase, lamins, and nuclear variants of cytoplasmic proteins such as actin are examples of nuclear antigens.

In a third embodiment, protein chimeras consisting of a nuclear protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the nuclear domain. Examples of these proteins are many of the proteins involved in maintaining DNA structure and function. Histones, DNA polymerase, RNA polymerase, lamins, and nuclear variants of cytoplasmic proteins such as actin are examples of nuclear proteins.

Mitochondrial labeling

In one embodiment, membrane permeant mitochondrial-specific luminescent reagents (Molecular Probes, Inc.) are used to label the mitochondria of living and fixed cells. These reagents include rhodamine 123, tetramethyl rosamine, JC-1, and the MitoTracker reactive dyes.

In a second embodiment, antibodies against mitochondrial antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label mitochondrial components that are localized in specific mitochondrial domains. Examples of these components are the macromolecules involved in maintaining mitochondrial DNA structure and function. DNA, RNA, histones, DNA polymerase, RNA polymerase, and mitochondrial variants of cytoplasmic macromolecules such as mitochondrial tRNA and rRNA are examples mitochondrial antigens. Other examples of mitochondrial antigens are the components of the oxidative phosphorylation system found in the mitochondria (*e.g.*, cytochrome c, cytochrome c oxidase, and succinate dehydrogenase).

In a third embodiment, protein chimeras consisting of a mitochondrial protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the mitochondrial domain. Examples of these components are the macromolecules involved in maintaining mitochondrial DNA structure and function. Examples include histones, DNA polymerase, RNA polymerase, and the components of the oxidative phosphorylation system found in the mitochondria (*e.g.*, cytochrome c, cytochrome c oxidase, and succinate dehydrogenase).

Endoplasmic reticulum labeling

In one embodiment, membrane permeant endoplasmic reticulum-specific luminescent reagents (Molecular Probes, Inc.) are used to label the endoplasmic reticulum of living and fixed cells. These reagents include short chain carbocyanine dyes (*e.g.*, DiOC₆ and DiOC₃), long chain carbocyanine dyes (*e.g.*, DiIC₁₆ and DiIC₁₈), and luminescently labeled lectins such as concanavalin A.

In a second embodiment, antibodies against endoplasmic reticulum antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label endoplasmic reticulum components that are localized in specific endoplasmic reticulum domains. Examples of these components are the macromolecules involved in the fatty acid elongation systems, glucose-6-phosphatase, and HMG CoA-reductase.

In a third embodiment, protein chimeras consisting of a endoplasmic reticulum protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the endoplasmic reticulum domain. Examples of these components are the macromolecules involved in the fatty acid elongation systems, glucose-6-phosphatase, and HMG CoA-reductase.

Golgi labeling

In one embodiment, membrane permeant Golgi-specific luminescent reagents (Molecular Probes, Inc.) are used to label the Golgi of living and fixed cells. These reagents include luminescently labeled macromolecules such as wheat germ agglutinin and Brefeldin A as well as luminescently labeled ceramide.

In a second embodiment, antibodies against Golgi antigens (Sigma Chemical Co.; Molecular Probes, Inc.; Caltag Antibody Co.) are used to label Golgi components that are localized in specific Golgi domains. Examples of these components are N-acetylglucosamine phosphotransferase, Golgi-specific phosphodiesterase, and mannose-6-phosphate receptor protein.

In a third embodiment, protein chimeras consisting of a Golgi protein genetically fused to an intrinsically luminescent protein such as the green fluorescent protein, or mutants thereof, are used to label the Golgi domain. Examples of these components are N-acetylglucosamine phosphotransferase, Golgi-specific phosphodiesterase, and mannose-6-phosphate receptor protein.

While many of the examples presented involve the measurement of single cellular processes, this is again intended for purposes of illustration only. Multiple parameter high-content screens can be produced by combining several single parameter screens into a multiparameter high-content screen or by adding cellular parameters to any existing high-content screen. Furthermore, while each example is described as being based on either live or fixed cells, each high-content screen can be designed to be used with both live and fixed cells.

Those skilled in the art will recognize a wide variety of distinct screens that can be developed based on the disclosure provided herein. There is a large and growing list of known biochemical and molecular processes in cells that involve translocations or reorganizations of specific components within cells. The signaling pathway from the cell surface to target sites within the cell involves the translocation of plasma membrane-associated proteins to the cytoplasm. For example, it is known that one of the src family of protein tyrosine kinases, pp60c-src (Walker et al (1993), *J. Biol. Chem.* 268:19552-19558) translocates from the plasma membrane to the cytoplasm upon stimulation of fibroblasts with platelet-derived growth factor (PDGF). Additionally, the targets for screening can themselves be converted into fluorescence-based reagents that report molecular changes including ligand-binding and post-translocational modifications.

Example 10. Protease Biosensors

(1) Background

As used herein, the following terms are defined as follows:

- Reactant – the parent biosensor that interacts with the proteolytic enzyme.
- Product – the signal-containing proteolytic fragment(s) generated by the interaction of the reactant with the enzyme.
- Reactant Target Sequence – an amino acid sequence that imparts a restriction on the cellular distribution of the reactant to a particular subcellular domain of the cell.
- Product Target Sequence – an amino acid sequence that imparts a restriction on the cellular distribution of the signal-containing product(s) of the targeted enzymatic reaction to a particular subcellular domain of the cell. If the product is initially localized within a membrane bound compartment, then the Product Target

Sequence must incorporate the ability to export the product out of the membrane-bound compartment. A bi-functional sequence can be used, which first moves the product out of the membrane-bound compartment, and then targets the product to the final compartment. In general, the same amino acid sequences can act as either
5 or both reactant target sequences and product target sequences. Exceptions to this include amino acid sequences which target the nuclear envelope, Golgi apparatus, endoplasmic reticulum, and which are involved in farnesylation, which are more suitable as reactant target sequences.

- Protease Recognition Site – an amino acid sequence that imparts specificity by
10 mimicking the substrate, providing a specific binding and cleavage site for a protease. Although typically a short sequence of amino acids representing the minimal cleavage site for a protease (e.g. DEVD for caspase-3, Villa, P., S.H. Kaufmann, and W.C. Earnshaw. 1997. Caspases and caspase inhibitors. *Trends Biochem Sci.* 22:388-93), greater specificity may be established by using a longer
15 sequence from an established substrate.

- Compartment – any cellular sub-structure or macromolecular component of the cell, whether it is made of protein, lipid, carbohydrate, or nucleic acid. It could be a macromolecular assembly or an organelle (a membrane delimited cellular component). Compartments include, but are not limited to, cytoplasm, nucleus,
20 nucleolus, inner and outer surface of nuclear envelope, cytoskeleton, peroxisome, endosome, lysosome, inner leaflet of plasma membrane, outer leaflet of plasma membrane, outer leaflet of mitochondrial membrane, inner leaflet of mitochondrial membrane, Golgi, endoplasmic reticulum, or extracellular space.

Signal – an amino acid sequence that can be detected. This includes, but is not
25 limited to inherently fluorescent proteins (e.g. Green Fluorescent Protein), cofactor-requiring fluorescent or luminescent proteins (e.g. phycobiliproteins or luciferases), and epitopes recognizable by specific antibodies or other specific natural or unnatural binding probes, including but not limited to dyes, enzyme cofactors and engineered binding molecules, which are fluorescently or luminescently labeled.
30 Also included are site-specifically labeled proteins that contain a luminescent dye. Methodology for site-specific labeling of proteins includes, but is not limited to, engineered dye-reactive amino acids (Post, et al., *J. Biol. Chem.* 269:12880-12887

(1994)), enzyme-based incorporation of luminescent substrates into proteins (Buckler, et al., *Analyt. Biochem.* 209:20-31 (1993); Takashi, *Biochemistry.* 27:938-943 (1988)), and the incorporation of unnatural labeled amino acids into proteins (Noren, et al., *Science.* 244:182-188 (1989)).

- 5 • Detection – a means for recording the presence, position, or amount of the signal. The approach may be direct, if the signal is inherently fluorescent, or indirect, if, for example, the signal is an epitope that must be subsequently detected with a labeled antibody. Modes of detection include, but are not limited to, the spatial position of fluorescence, luminescence, or phosphorescence: (1) intensity; (2) polarization; (3) 10 lifetime; (4) wavelength; (5) energy transfer; and (6) recovery after photobleaching.

The basic principle of the protease biosensors of the present invention is to spatially separate the reactants from the products generated during a proteolytic reaction. The separation of products from reactants occurs upon proteolytic cleavage of the protease recognition site within the biosensor, allowing the products to bind to, 15 diffuse into, or be imported into compartments of the cell different from those of the reactant. This spatial separation provides a means of quantitating a proteolytic process directly in living or fixed cells. Some designs of the biosensor provide a means of restricting the reactant (uncleaved biosensor) to a particular compartment by a protein sequence (“reactant target sequence”) that binds to or imports the biosensor into a 20 compartment of the cell. These compartments include, but are not limited to any cellular substructure, macromolecular cellular component, membrane-limited organelles, or the extracellular space. Given that the characteristics of the proteolytic reaction are related to product concentration divided by the reactant concentration, the spatial separation of products and reactants provides a means of uniquely quantitating 25 products and reactants in single cells, allowing a more direct measure of proteolytic activity.

The molecular-based biosensors may be introduced into cells via transfection and the expressed chimeric proteins analyzed in transient cell populations or stable cell lines. They may also be pre-formed, for example by production in a prokaryotic or 30 eukaryotic expression system, and the purified protein introduced into the cell via a number of physical mechanisms including, but not limited to, micro-injection, scrape loading, electroporation, signal-sequence mediated loading, etc.

Measurement modes may include, but are not limited to, the ratio or difference in fluorescence, luminescence, or phosphorescence: (a) intensity; (b) polarization; or (c) lifetime between reactant and product. These latter modes require appropriate spectroscopic differences between products and reactants. For example, cleaving a reactant containing a limited-mobile signal into a very small translocating component and a relatively large non-translocating component may be detected by polarization. Alternatively, significantly different emission lifetimes between reactants and products allow detection in imaging and non-imaging modes.

One example of a family of enzymes for which this biosensor can be constructed to report activity is the caspases. Caspases are a class of proteins that catalyze proteolytic cleavage of a wide variety of targets during apoptosis. Following initiation of apoptosis, the Class II "downstream" caspases are activated and are the point of no return in the pathway leading to cell death, resulting in cleavage of downstream target proteins. In specific examples, the biosensors described here were engineered to use nuclear translocation of cleaved GFP as a measurable indicator of caspase activation. Additionally, the use of specific recognition sequences that incorporate surrounding amino acids involved in secondary structure formation in naturally occurring proteins may increase the specificity and sensitivity of this class of biosensor.

Another example of a protease class for which this biosensor can be constructed to report activity is zinc metalloproteases. Two specific examples of this class are the biological toxins derived from *Clostridial* species (*C. botulinum* and *C. tetani*) and *Bacillus anthracis*. (Herreros et al. *In The Comprehensive Sourcebook of Bacterial Protein Toxins*. J.E. Alouf and J.H. Freer, Eds. 2nd edition, San Diego, Academic Press, 1999; pp 202-228.) These bacteria express and secrete zinc metalloproteases that enter eukaryotic cells and specifically cleave distinct target proteins. For example, the anthrax protease from *Bacillus anthracis* is delivered into the cytoplasm of target cells via an accessory pore-forming protein, where its proteolytic activity inactivates the MAP-kinase signaling cascade through cleavage of mitogen activated protein kinase kinases 1 or 2 (MEK1 or MEK2). (Leppia, S.A. *In The Comprehensive Sourcebook of Bacterial Protein Toxins*. J.E. Alouf and J.H. Freer, Eds. 2nd edition, San Diego, Academic Press, 1999; pp243-263.) The toxin biosensors described here take

advantage of the natural subcellular localization of these and other target proteins to achieve reactant targeting. Upon cleavage, the signal (with or without a product target sequence) is separated from the reactant to create a high-content biosensor.

One of skill in the art will recognize that the protein biosensors of this aspect of the invention can be adapted to report the activity of any member of the caspase family of proteases, as well as any other protease, by a substitution of the appropriate protease recognition site in any of the constructs (see **Figure 29B**). These biosensors can be used in high-content screens to detect in vivo activation of enzymatic activity and to identify specific activity based on cleavage of a known recognition motif. This screen can be used for both live cell and fixed end-point assays, and can be combined with additional measurements to provide a multi-parameter assay.

Thus, in another aspect the present invention provides recombinant nucleic acids encoding a protease biosensor, comprising:

- a. a first nucleic acid sequence that encodes at least one detectable polypeptide signal;
- b. a second nucleic acid sequence that encodes at least one protease recognition site, wherein the second nucleic acid sequence is operatively linked to the first nucleic acid sequence that encodes the at least one detectable polypeptide signal; and
- c. a third nucleic acid sequence that encodes at least one reactant target sequence, wherein the third nucleic acid sequence is operatively linked to the second nucleic acid sequence that encodes the at least one protease recognition site.

In this aspect, the first and third nucleic acid sequences are separated by the second nucleic acid sequence, which encodes the protease recognition site.

In a further embodiment, the recombinant nucleic acid encoding a protease biosensor comprises a fourth nucleic acid sequence that encodes at least one product target sequence, wherein the fourth nucleic acid sequence is operatively linked to the first nucleic acid sequence that encodes the at least one detectable polypeptide signal.

In a further embodiment, the recombinant nucleic acid encoding a protease biosensor comprises a fifth nucleic acid sequence that encodes at least one detectable

polypeptide signal, wherein the fifth nucleic acid sequence is operatively linked to the third nucleic acid sequence that encodes the reactant target sequence.

In a preferred embodiment, the detectable polypeptide signal is selected from the group consisting of fluorescent proteins, luminescent proteins, and sequence epitopes. In a most preferred embodiment, the first nucleic acid encoding a polypeptide sequence comprises a sequence selected from the group consisting of SEQ ID NOS: 35, 37, 39, 41, 43, 45, 47, 49, and 51.

In another preferred embodiment, the second nucleic acid encoding a protease recognition site comprises a sequence selected from the group consisting of SEQ ID NOS: 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, and 121. In another preferred embodiment, the third nucleic acid encoding a reactant target sequence comprises a sequence selected from the group consisting of SEQ ID NOS: 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, and 151.

In a most preferred embodiment, the recombinant nucleic acid encoding a protease biosensor comprises a sequence substantially similar to sequences selected from the group consisting of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, and 33.

In another aspect, the present invention provides a recombinant expression vector comprising nucleic acid control sequences operatively linked to the above-described recombinant nucleic acids. In a still further aspect, the present invention provides genetically engineered host cells that have been transfected with the recombinant expression vectors of the invention.

In another aspect, the present invention provides recombinant protease biosensors comprising

- a. a first domain comprising at least one detectable polypeptide signal;
- b. a second domain comprising at least one protease recognition site; and
- c. a third domain comprising at least one reactant target sequence; wherein the first domain and the third domain are separated by the second domain.

Inherent in this embodiment is the concept that the reactant target sequence restricts the cellular distribution of the reactant, with redistribution of the product occurring after activation (ie: protease cleavage). This redistribution does not require a complete sequestration of products and reactants, as the product distribution can partially overlap the reactant distribution in the absence of a product targeting signal (see below).

In a preferred embodiment, the recombinant protease biosensor further comprises a fourth domain comprising at least one product target sequence, wherein the fourth domain and the first domain are operatively linked and are separated from the third domain by the second domain. In another embodiment, the recombinant protease biosensor further comprises a fifth domain comprising at least one detectable polypeptide signal, wherein the fifth domain and the third domain are operatively linked and are separated from the first domain by the second domain.

In a preferred embodiment, the detectable polypeptide signal domain (first or fifth domain) is selected from the group consisting of fluorescent proteins, luminescent proteins, and sequence epitopes. In a most preferred embodiment, the detectable polypeptide signal domain comprises a sequence selected from the group consisting of SEQ ID NOS:36, 38, 40, 42, 44, 46, 48, 50, and 52.

In another preferred embodiment, the second domain comprising a protease recognition site comprises a sequence selected from the group consisting of SEQ ID NOS:54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, and 122. In another preferred embodiment, the reactant and/or target sequence domains comprise a sequence selected from the group consisting of SEQ ID NOS:124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, and 152.

In a most preferred embodiment, the recombinant protease biosensor comprises a sequence substantially similar to sequences selected from the group consisting of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, and 34.

In a still further embodiment, the present invention provides methods and kits for automated analysis of cells, comprising using cells that possess the protease biosensors of the invention to identify compounds that affect protease activity. The

method can be combined with the other methods of the invention in a variety of possible multi-parametric assays.

In these various embodiments, the basic protease biosensor is composed of multiple domains, including at least a first detectable polypeptide signal domain, at least one reactant target domain, and at least one protease recognition domain, wherein the detectable signal domain and the reactant target domain are separated by the protease recognition domain. Thus, the exact order of the domains in the molecule is not generally critical, so long as the protease recognition domain separates the reactant target and first detectable signal domain. For each domain, one or more one of the specified recognition sequences is present.

In some cases, the order of the domains in the biosensor may be critical for appropriate targeting of product(s) and/or reactant to the appropriate cellular compartment(s). For example, the targeting of products or reactants to the peroxisome requires that the peroxisomal targeting domain comprise the last three amino acids of the protein. Determination of those biosensor in which the relative placement of targeting domains within the biosensor is critical can be determined by one of skill in the art through routine experimentation.

Some examples of the basic organization of domains within the protease biosensor are shown in Figure 30. One of skill in the art will recognize that any one of a wide variety of protease recognition sites, product target sequences, polypeptide signals, and/or product target sequences can be used in various combinations in the protein biosensor of the present invention, by substituting the appropriate coding sequences into the multi-domain construct. Non-limiting examples of such alternative sequences are shown in Figure 29A-29C. Similarly, one of skill in the art will recognize that modifications, substitutions, and deletions can be made to the coding sequences and the amino acid sequence of each individual domain within the biosensor, while retaining the function of the domain. Such various combinations of domains and modifications, substitutions and deletions to individual domains are within the scope of the invention.

As used herein, the term "coding sequence" or a sequence which "encodes" a particular polypeptide sequence, refers to a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vitro

or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA,
5 genomic DNA sequences from prokaryotic or eukaryotic DNA, and synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

As used herein, the term DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription
10 termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell. Not all of these control sequences need always be present in a recombinant vector so long as the DNA sequence of interest is capable of being transcribed and translated appropriately.

As used herein, the term "operatively linked" refers to an arrangement of
15 elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operatively linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the
20 expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operatively linked" to the coding sequence.

Furthermore, a nucleic acid coding sequence is operatively linked to another
25 nucleic acid coding sequences when the coding region for both nucleic acid molecules are capable of expression in the same reading frame. The nucleic acid sequences need not be contiguous, so long as they are capable of expression in the same reading frame. Thus, for example, intervening coding regions can be present between the specified nucleic acid coding sequences, and the specified nucleic acid coding regions can still be
30 considered "operatively linked".

The intervening coding sequences between the various domains of the biosensors can be of any length so long as the function of each domain is retained.

Generally, this requires that the two-dimensional and three-dimensional structure of the intervening protein sequence does not preclude the binding or interaction requirements of the domains of the biosensor, such as product or reactant targeting, binding of the protease of interest to the biosensor, fluorescence or luminescence of the detectable polypeptide signal, or binding of fluorescently labeled epitope-specific antibodies.

One case where the distance between domains of the protease biosensor is important is where the goal is to create a fluorescence resonance energy transfer pair. In this case, the FRET signal will only exist if the distance between the donor and acceptor is sufficiently small as to allow energy transfer (Tsien, Heim and Cubbit, WO 97/28261). The average distance between the donor and acceptor moieties should be between 1 nm and 10 nm with a preference of between 1 nm and 6 nm. This is the physical distance between donor and acceptor. The intervening sequence length can vary considerably since the three dimensional structure of the peptide will determine the physical distance between donor and acceptor.

"Recombinant expression vector" includes vectors that operatively link a nucleic acid coding region or gene to any promoter capable of effecting expression of the gene product. The promoter sequence used to drive expression of the protease biosensor may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive). The expression vector must be replicable in the host organisms either as an episome or by integration into host chromosomal DNA. In a preferred embodiment, the expression vector comprises a plasmid. However, the invention is intended to include any other suitable expression vectors, such as viral vectors.

The phrase "substantially similar" is used herein in reference to the nucleotide sequence of DNA, or the amino acid sequence of protein, having one or more conservative or non-conservative variations from the protease biosensor sequences disclosed herein, including but not limited to deletions, additions, or substitutions wherein the resulting nucleic acid and/or amino acid sequence is functionally equivalent to the sequences disclosed and claimed herein. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same protease biosensor as the nucleic acid and amino acid compositions disclosed and

claimed herein. For example, functionally equivalent DNAs encode protease biosensors that are the same as those disclosed herein or that have one or more conservative amino acid variations, such as substitutions of non-polar residues for other non-polar residues or charged residues for similarly charged residues, or addition to/deletion from regions of the protease biosensor not critical for functionality. These changes include those recognized by those of skill in the art as substitutions, deletions, and/or additions that do not substantially alter the tertiary structure of the protein.

As used herein, substantially similar sequences of nucleotides or amino acids share at least about 70%-75% identity, more preferably 80-85% identity, and most preferably 90-95% identity. It is recognized, however, that proteins (and DNA or mRNA encoding such proteins) containing less than the above-described level of homology (due to the degeneracy of the genetic code) or that are modified by conservative amino acid substitutions (or substitution of degenerate codons) are contemplated to be within the scope of the present invention.

The term "heterologous" as it relates to nucleic acid sequences such as coding sequences and control sequences, denotes sequences that are not normally associated with a region of a recombinant construct, and/or are not normally associated with a particular cell. Thus, a "heterologous" region of a nucleic acid construct is an identifiable segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, a host cell transformed with a construct which is not normally present in the host cell would be considered heterologous for purposes of this invention.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutscher, ed., (1990) Academic Press, Inc.); *PCR*

Protocols: A Guide to Methods and Applications (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion
5 1998 Catalog (Ambion, Austin, TX).

The biosensors of the present invention are constructed and used to transfect host cells using standard techniques in the molecular biological arts. Any number of such techniques, all of which are within the scope of this invention, can be used to generate protease biosensor-encoding DNA constructs and genetically transfected host
10 cells expressing the biosensors. The non-limiting examples that follow demonstrate one such technique for constructing the biosensors of the invention.

EXAMPLE OF PROTEASE BIOSENSOR CONSTRUCTION AND USE:

In the following examples, caspase-specific biosensors with specific product
15 target sequences have been constructed using sets of 4 primers (2 sense and 2 antisense). These primers have overlap regions at their termini, and are used for PCR via a primer walking technique. (Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) The two sense primers were chosen to start from the 5'
20 polylinker (BspI) of the GFP-containing vector (Clontech, California) to the middle of the designed biosensor sequence. The two antisense primers start from a 3' GFP vector site (Bam HI), and overlap with the sense primers by 12 nucleotides in the middle.

PCR conditions were as follows: 94°C for 30 seconds for denaturation, 55°C for 30 seconds for annealing, and 72°C for 30 seconds for extension for 15 cycles. The
25 primers have restriction endonuclease sites at both ends, facilitating subsequent cloning of the resulting PCR product.

The resulting PCR product was gel purified, cleaved at BspE1 and BamH1 restriction sites present in the primers, and the resulting fragment was gel purified. Similarly, the GFP vector (Clontech, San Francisco, CA) was digested at BspE1 and
30 BamH1 sites in the polylinker. Ligation of the GFP vector and the PCR product was performed using standard techniques at 16°C overnight. *E. coli* cells were transfected

with the ligation mixtures using standard techniques. Transformed cells were selected on LB-agar with an appropriate antibiotic.

Cells and transfections. For DNA transfection, BHK cells and MCF-7 cells were cultured to 50-70% confluence in 6 well plates containing 3 ml of minimal Eagle's medium (MEM) with 10% fetal calf serum, 1 mM L-glutamine, 50 µg/ml streptomycin, 50 µg/ml penicillin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10 µg/ml of bovine insulin (for MCF-7 cell only) at 37 °C in a 5% CO₂ incubator for about 36 hours. The cells were washed with serum free MEM media and incubated for 5 hours with 1 ml of transfection mixture containing 1 µg of the appropriate plasmid and 4 µg of lipofectimine (BRL) in the serum free MEM media. Subsequently, the transfection medium was removed and replaced with 3 ml of normal culture media. The transfected cells were maintained in growth medium for at least 16 hours before performing selection of the stable cells based on standard molecular biology methods (Ausubel. et al 1995).

Apoptosis assay. For apoptosis assays, the cells (BHK, MCF-7) stably transfected with the appropriate protease biosensor expression vector were plated on tissue culture treated 96-well plates at 50-60% confluence and cultured overnight at 37°C, 5% CO₂. Varying concentrations of cis-platin, staurosporine, or paclitaxel in normal culture media were freshly prepared from stock and added to cell culture dishes to replace the old culture media. The cells were then observed with the cell screening system of the present invention at the indicated time points either as live cell experiments or as fixed end-point experiments.

1. Construction of 3-domain protease biosensors

a. Caspase-3 biosensor with an annexin II reactant targeting domain (pljkGFP).

The design of this biosensor is outlined in Figure 31, and its sequence is shown in SEQ ID NO:1 and 2.

Primers for Caspase 3, Product target sequence = none (CP3GFP-CYTO):

- 1) TCA TCA TCC GGA GCT GGA GCC GGA GCT GGC CGA TCG GCT GTT
AAA TCT GAA GGA AAG AGA AAG TGT GAC GAA GTT GAT GGA ATT
5 GAT GAA GTA GCA (SEQ ID NO:153)
- 2)GAA GAA GGA TCC GGC ACT TGG GGG TGT AGA ATG AAC ACC
CTC CAA GCT GAG CTT GCA CAG GAT TTC GTG GAC AGT AGA
CAT AGT ACT TGC TAC TTC ATC (SEQ ID NO:154)
- 3) TCA TCA TCC GGA GCT GGA (SEQ ID NO:155)
- 10 4) GAA GAA GGA TCC GGC ACT (SEQ ID NO:156)

This biosensor is restricted to the cytoplasm by the reactant target sequence. The reactant target sequence is the annexin II cytoskeletal binding domain (MSTVHEILCKLSLEGVHSTPPSA) (SEQ ID NO:124) (Figure 29C) (Eberhard et
15 al. 1997. *Mol. Biol. Cell* 8:293a). The enzyme recognition site corresponds to two copies of the amino acid sequence DEVD (SEQ ID NO:60) (Figure 29B), which serves as the recognition site of caspase-3. Other examples with different numbers of protease recognition sites and/or additional amino acids from a naturally occurring protease recognition site are shown below. The signal domain is EGFP (SEQ ID
20 NO:46) (Figure 29A) (Clontech, California). The parent biosensor (the reactant) is restricted to the cytoplasm by binding of the annexin II domain to the cytoskeleton, and is therefore excluded from the nucleus. Upon cleavage of the protease recognition site by caspase 3, the signal domain (EGFP) is released from the reactant targeting domain (annexin II), and is distributed throughout the whole volume of the cell, because it lacks
25 any specific targeting sequence and is small enough to enter the nucleus passively. (Fig 32)

The biosensor response is measured by quantitating the effective cytoplasm-to-nuclear translocation of the signal (see above). Measurement of the response is by one of several modes, including integrated or average nuclear region intensity, the ratio or
30 difference of the integrated or average cytoplasm intensity to integrated or average nuclear intensity. The nucleus is defined using a DNA-specific dye, such as Hoechst 33342.

This biosensor provides a measure of the proteolytic activity around the annexin II cytoskeleton binding sites within the cell. Given the dispersed nature of the cytoskeleton and the effectively diffuse state of cytosolic enzymes, this provides an effective measure of the cytoplasm in general.

5

Results & Discussion:

Fig 32 illustrates images before and after stimulation of apoptosis by cis-platin in BHK cells, transfected with the caspase 3 biosensor. The images clearly illustrate accumulation of fluorescence in the nucleus. Generation of the spatial change in fluorescence is non-reversible and thus the timing of the assay is flexible. Controls for this biosensor include using a version in which the caspase-3-specific site has been omitted. In addition, disruption of the cytoskeleton with subsequent cell rounding did not produce the change in fluorescence distribution. Our experiments demonstrate the correlation of nuclear condensation with activation of caspase activity. We have also tested this biosensor in MCF-7 cells. A recent report measured a peak response in caspase-3 activity 6 h after stimulation of MCF-7 cells with etoposide accompanied by cleavage of PARP (Benjamin et al. 1998. *Mol Pharmacol.* 53:446-50). However, another recent report found that MCF-7 cells do not possess caspase-3 activity and, in fact, the caspase-3 gene is functionally deleted (Janicke et al. 1998. *J Biol Chem.* 273:9357-60). Caspase-3 activity was not detected with the caspase biosensor in MCF-7 cells after a 15 h treatment with 100 μ M etoposide.

Janicke et al., (1998) also indicated that many of the conventional substrates of caspase-3 were cleaved in MCF-7 cells upon treatment with staurosporine. Our experiments demonstrate that caspase activity can be measured using the biosensor in MCF-7 cells when treated with staurosporine. The maximum magnitude of the activation by staurosporine was approximately one-half that demonstrated with cis-platin in BHK cells. This also implies that the current biosensor, although designed to be caspase-3-specific, is indeed specific for a class of caspases rather than uniquely specific for caspase-3. The most likely candidate is caspase-7 (Janicke et al., 1998). These experiments also demonstrated that the biosensor can be used in multiparameter experiments, with the correlation of decreases in mitochondrial membrane potential, nuclear condensation, and caspase activation.

We have specifically tested the effects of paclitaxel on caspase activation using the biosensor. Caspase activity in BHK and MCF-7 cells was stimulated by paclitaxel. It also appears that caspase activation occurred after nuclear morphology changes. One caveat is that, based on the above discussions, the caspase activity reported by the biosensor in this assay is likely to be due to the combination of caspase-3 and, at least, caspase-7 activity.

Consistent with the above results using staurosporine stimulation on MCF-7 cells, paclitaxel also stimulated the activation of caspase activity. The magnitude was similar to that of staurosporine. This experiment used a much narrower range of paclitaxel than previous experiments where nuclear condensation appears to dominate the response.

b. Caspase biosensor with the microtubule associated protein 4 (MAP4) projection domain (CP8GFPNLS-SIZEPROJ)

Another approach for restricting the reactant to the cytoplasm is to make the biosensor too large to penetrate the nuclear pores. Cleavage of such a biosensor liberates a product capable of diffusing into the nucleus.

The additional size required for this biosensor is provided by using the projection domain of MAP4 (SEQ ID NO:142) (Figure 29C) (CP8GFPNLS-SIZEPROJ). The projection domain of MAP4 does not interact with microtubules on its own, and, when expressed, is diffusely distributed throughout the cytoplasm, but is excluded from the nucleus due to its size (~120 kD). Thus, this biosensor is distinct from the one using the full length MAP4 sequence. (see below) One of skill in the art will recognize that many other such domains could be substituted for the MAP4 projection domain, including but not limited to multiple copies of any GFP or one or more copies of any other protein that lacks an active NLS and exceeds the maximum size for diffusion into the nucleus (approximately 60 kD; Alberts, B., Bray, D., Raff, M., Roberts, K., Watson, J.D. (Eds.) Molecular Biology of the Cell, third edition, New York: Garland publishing, 1994. pp 561-563). The complete sequence of the resulting biosensor is shown in SEQ ID NO: 3-4. A similar biosensor with a different protease recognition domain is shown in SEQ ID NO:5-6.

c. Caspase biosensor with a nuclear export signal

Another approach for restricting the reactant to the cytoplasm is to actively restrict the reactant from the nucleus by using a nuclear export signal. Cleavage of such a biosensor liberates a product capable of diffusing into the nucleus.

5 The *Bacillus anthracis* bacterium expresses a zinc metalloprotease protein complex called anthrax protease. Human mitogen activated protein kinase kinase 1 (MEK 1) (Seeger et al., J. Biol. Chem. 267:25628-25631, 1992) possesses an anthrax protease recognition site (amino acids 1-13) (SEQ ID NO:102) (Figure 29B) that is cleaved after amino acid 8, as well as a nuclear export signal at amino acids 32-44
10 (SEQ ID NO:140) (Figure 29C). Human MEK 2 (Zheng and Guan, J. Biol. Chem. 268:11435-11439, 1993) possesses an anthrax protease recognition site comprising amino acid residues 1-16 (SEQ ID NO:104) (Figure 29B) and a nuclear export signal at amino acids 36-48. (SEQ ID NO:148) (Figure 29C).

The anthrax protease biosensor comprises Fret25 (SEQ ID NO:48) (Figure
15 29A) as the signal, the anthrax protease recognition site, and the nuclear export signal from MEK 1 or MEK2. (SEQ ID NOS: 7-8 (MEK1); 9-10 (MEK2)) The intact biosensor will be retained in the cytoplasm by virtue of this nuclear export signal (eg., the reactant target site). Upon cleavage of the fusion protein by anthrax protease, the NES will be separated from the GFP allowing the GFP to diffuse into the nucleus.

2. Construction of 4- and 5-domain biosensors

For all of the examples presented above for 3-domain protease biosensors, a product targeting sequence, including but not limited to those in Figure 29C, such as a nuclear localization sequence (NLS), can be operatively linked to the signal sequence,
25 and thus cause the signal sequence to segregate from the reactant target domain after proteolytic cleavage. Addition of a second detectable signal domain, including but not limited to those in Figure 29A, operatively linked with the reactant target domain is also useful in allowing measurement of the reaction by multiple means. Specific examples of such biosensors are presented below.

a. 4 domain biosensors

1. Caspase biosensors with nuclear localization sequences

(pcas3nlsGFP; CP3GFPNLS-CYTO):

The design of the biosensor is outlined in **Figure 33**, and its sequence is shown in **SEQ ID NO:11-12**. PCR and cloning procedures were performed as described above, except that the following oligonucleotides were used:

5 **Primers for Caspase 3, Product target sequence = NLS (CP3GFPNLS-CYTO) :**

- 1) TCA TCA TCC GGA AGA AGG AAA CGA CAA AAG CGA TCG GCT
GTT AAA TCT GAA GGA AAG AGA AAG TGT GAC GAA GTT GAT GGA
ATT GAT GAA GTA GCA (SEQ ID NO:157)
- 10 2) GAA GAA GGA TCC GGC ACT TGG GGG TGT AGA ATG AAC ACC
CTC CAA GCT GAG CTT GCA CAG GAT TTC GTG GAC AGT AGA
CAT AGT ACT TGC TAC TTC ATC (SEQ ID NO:154)
- 3) TCA TCA TCC GGA AGA AGG (SEQ ID NO:158)
- 4) GAA GAA GGA TCC GGC ACT (SEQ ID NO:156)

15

This biosensor is similar to that shown in **SEQ ID NO:2** except upon recognition and cleavage of the protease recognition site, the product is released and the signal accumulates specifically in the nucleus due to the presence of a nuclear localization sequence, RRKRQK (**SEQ ID NO:128**) (**Figure 29C**)(Briggs et al., J. Biol. Chem. 273:22745, 1998) attached to the signal. A specific benefit of this

20 construct is that the products are clearly separated from the reactants. The reactants remain in the cytoplasm, while the product of the enzymatic reaction is restricted to the nuclear compartment. The response is measured by quantitating the effective cytoplasm-to-nuclear translocation of the signal, as described above.

25

With the presence of both product and reactant targeting sequences in the parent biosensor, the reactant target sequence should be dominant prior to activation (e.g., protease cleavage) of the biosensor. One way to accomplish this is by masking the product targeting sequence in the parent biosensor until after protease cleavage. In one such example, the product target sequence is functional only when relatively near the

30 end of a polypeptide chain (ie: after protease cleavage). Alternatively, the biosensor may be designed so that its tertiary structure masks the function of the target sequence until after protease cleavage. Both of these approaches include comparing targeting

sequences with different relative strengths for targeting. Using the example of the nuclear localization sequence (NLS) and annexin II sequences, different strengths of NLS have been tried with clone selection based on cytoplasmic restriction of the parent biosensor. Upon activation, the product targeting sequence will naturally dominate the
 5 localization of its associated detectable sequence domain because it is then separated from the reactant targeting sequence.

An added benefit of using this biosensor is that the product is targeted, and thus concentrated, into a smaller region of the cell. Thus, smaller amounts of product are detectable due to the increased concentration of the product. This concentration effect
 10 is relatively insensitive to the cellular concentration of the reactant. The signal-to-noise ratio (SNR) of such a measurement is improved over the more dispersed distribution of biosensor #1.

Similar biosensors that incorporate either the caspase 6 (SEQ ID NO:66) (Figure 29B) or the caspase 8 protease recognition sequence (SEQ ID NO:74) (Figure
 15 29B) can be made using the methods described above, but using the following primer sets:

Primers for Caspase 6, Product target sequence = NLS (CP6GFPNLS-CYTO)

- 1) TCA TCA TCC GGA AGA AGG AAA CGA CAA AAG CGA TCG
 20 ACA AGA CTT GTT GAA ATT GAC AAC (SEQ ID NO:159)
- 2) GAA GAA GGA TCC GGC ACT TGG GGG TGT AGA ATG AAC
 ACC CTC CAA GCT GAG CTT GCA CAG GAT TTC GTG GAC
 AGT AGA CAT AGT ACT GTT GTC AAT TTC (SEQ ID NO:160)
- 25 3) TCA TCA TCC GGA AGA AGG (SEQ ID NO:158)
- 4) GAA GAA GGA TCC GGC ACT (SEQ ID NO:156)

Primers for Caspase 8, Product target sequence = NLS (CP8GFPNLS-CYTO)

- 1) TCA TCA TCC GGA AGA AGG AAA CGA CAA AAG CGA TCG
 30 TAT CAA AAA GGA ATA CCA GTT GAA ACA GAC AGC GAA GAG
 CAA CCT TAT (SEQ ID NO:161)
- 2) GAA GAA GGA TCC GGC ACT TGG GGG TGT AGA ATG AAC ACC CTC

CAA GCT GAG CTT GCA CAG GAT TTC GTG GAC AGT AGA CAT AGT
ACT ATA AGG TTG CTC (SEQ ID NO:162)

3) TCA TCA TCC GGA AGA AGG (SEQ ID NO:158)

4) GAA GAA GGA TCC GGC ACT (SEQ ID NO:156)

5

The sequence of the resulting biosensors is shown in SEQ ID NO:13-14 (Caspase 6) and SEQ ID NO: 15-16 (Caspase 8). Furthermore, multiple copies of the protease recognition sites can be inserted into the biosensor, yielding the biosensors shown in SEQ ID NO: 17-18 (Caspase 3) and SEQ ID NO:19-20 (Caspase 8).

10

2. Caspase 3 biosensor with a second signal domain

An alternative embodiment employs a second signal domain operatively linked to the reactant target domain. In this example, full length MAP4 serves as the reactant target sequence. Upon recognition and cleavage, one product of the reaction, containing the reactant target sequence, remains bound to microtubules in the cytoplasm with its own unique signal, while the other product, containing the product target sequence, diffuses into the nucleus. This biosensor provides a means to measure two activities at once: caspase 3 activity using a translocation of GFP into the nucleus and microtubule cytoskeleton integrity in response to signaling cascades initiated during apoptosis, monitored by the MAP4 reactant target sequence.

20

The basic premise for this biosensor is that the reactant is tethered to the microtubule cytoskeleton by virtue of the reactant target sequence comprising the full length microtubule associated protein MAP4 (SEQ ID NO:152) (Figure 29C) In this case, a DEVD (SEQ ID NO:60) (Figure 29B) recognition motif is located between the EYFP signal (SEQ ID NO:44) (Figure 29A) operatively linked to the reactant target sequence, as well as the EBFP signal (SEQ ID NO:48) (Figure 29A) operatively linked to the C-terminus of MAP4. The resulting biosensor is shown in SEQ ID NO:21-22.

25

This biosensor can also include a product targeting domain, such as an NLS, operatively linked to the signal domain.

30

With this biosensor, caspase-3 cleavage still releases the N-terminal GFP, which undergoes translocation to the nucleus (directed there by the NLS). Also, the MAP4

fragment, which is still intact following proteolysis by caspase-3, continues to report on the integrity of the microtubule cytoskeleton during the process of apoptosis via the second GFP molecule fused to the C-terminus of the biosensor. Therefore, this single chimeric protein allows simultaneous analysis of caspase-3 activity and the polymerization state of the microtubule cytoskeleton during apoptosis induced by a variety of agents. This biosensor is also useful for analysis of potential drug candidates that specifically target the microtubule cytoskeleton, since one can determine whether a particular drug induced apoptosis in addition to affecting microtubules.

This biosensor potentially combines a unique signal for the reactant, fluorescence resonance energy transfer (FRET) from signal 2 to signal 1, and a unique signal localization for the product, nuclear accumulation of signal 1. The amount of product generated will also be indicated by the magnitude of the loss in FRET, but this will be a smaller SNR than the combination of FRET detection of reactant and spatial localization of the product.

FRET can occur when the emission spectrum of a donor overlaps significantly the absorption spectrum of an acceptor molecule. (dos Remedios, C.G., and P.D. Moens. 1995. Fluorescence resonance energy transfer spectroscopy is a reliable "ruler" for measuring structural changes in proteins. Dispelling the problem of the unknown orientation factor. *J Struct Biol.* 115:175-85; Emmanouilidou, E., A.G. Teschemacher, A.E. Pouli, L.I. Nicholls, E.P. Seward, and G.A. Rutter. 1999. Imaging Ca(2+) concentration changes at the secretory vesicle surface with a recombinant targeted cameleon. *Curr Biol.* 9:915-918.) The average physical distance between the donor and acceptor molecules should be between 1 nm and 10 nm with a preference of between 1 nm and 6 nm. The intervening sequence length can vary considerably since the three dimensional structure of the peptide will determine the physical distance between donor and acceptor. This FRET signal can be measured as (1) the amount of quenching of the donor in the presence of the acceptor, (2) the amount of acceptor emission when exciting the donor, and/or (3) the ratio between the donor and acceptor emission. Alternatively, fluorescent lifetimes of donor and acceptor could be measured.

This case adds value to the above FRET biosensor by nature of the existence of the reactant targeting sequence. This sequence allows the placement of the biosensor

into specific compartments of the cell for a more direct readout of activity in those compartments such as the inner surface of the plasma membrane.

The cytoplasmic second signal represents both original reactant plus one part of the product. The nuclear first signal represents another product of the reaction. Thus the enzymatic reaction has the added flexibility in that it can be represented as (1) nuclear intensity; (2) the nucleus /cytoplasm ratio; (3) the nucleus /cytoplasm FRET ratio; (4) cytoplasmic /cytoplasmic FRET ratio.

The present FRET biosensor design differs from previous FRET-based biosensors (see WO 97/28261; WO9837226) in that its signal measurement is based on spatial position rather than intensity. The products of the reaction are segregated from the reactants. It is this change in spatial position that is measured. The FRET-based biosensor is based on the separation, but not to another compartment, of a donor and acceptor pair. The intensity change is due to the physical separation of the donor and acceptor upon proteolytic cleavage. The disadvantages of FRET-based biosensors are (1) the SNR is rather low and difficult to measure, (2) the signal is not fixable. It must be recorded using living cells. Chemical fixation, for example with formaldehyde, cannot preserve both the parent and resultant signal; (3) the range of wavelengths are limiting and cover a larger range of the spectrum due to the presence of two fluorophores or a fluorophore and chromophore; (4) the construction has greater limitations in that the donor and acceptor must be precisely arranged to ensure that the distance falls within 1-10 nm.

Benefits of the positional biosensor includes: (1) ability to concentrate the signal in order to achieve a higher SNR. (2) ability to be used with either living or fixed cells; (3) only a single fluorescent signal is needed; (4) the arrangement of the domains of the biosensor is more flexible. The only limiting factor in the application of the positional biosensor is the need to define the spatial position of the signal which requires an imaging method with sufficient spatial resolution to resolve the difference between the reactant compartment and the product compartment.

One of skill in the art will recognize that this approach can be adapted to report any desired combination of activities by simply making the appropriate substitutions for the protease recognition sequence and the reactant target sequence, including but not limited to those sequences shown in Figure 29A-C.

3. Caspase 8 biosensor with a nucleolar localization domain (CP8GFPNUC-CYTO)

This approach (diagrammed in **Figure 34**) utilizes a biosensor for the detection of caspase-8 activity. In this biosensor, a nucleolar localization signal (RKRIPTYLKSCRRMKRSGFEMSRPIPSHLT) (SEQ ID NO:130) (**Figure 29C**) (Ueki et al., Biochem. Biophys. Res. Comm. 252:97-100, 1998) was used as the product target sequence, and made by PCR using the primers described below. The PCR product was digested with BspE1 and Pvu1 and gel purified. The vector and the PCR product were ligated as described above.

Primers for Caspase 8, Nucleolar localization signal (CP8GFPNUC-CYTO):

- 1) TCA TCA TCC GGA AGA AAA CGT ATA CGT ACT TAC CTC AAG
TCC TGC AGG CGG ATG AAA AGA (SEQ ID NO:163)
- 2) GAA GAA CGA TCG AGT AAG GTG GGA AGG AAT AGG TCG AGA
CAT CTC AAA ACC ACT TCT TTT CAT (SEQ ID NO:164)
- 3) TCA TCA TCC GGA AGA AAA (SEQ ID NO:165)
- 4) GAA GAA CGA TCG AGT AAG (SEQ ID NO:166)

The sequence of the resulting biosensor is shown in **SEQ ID NO: 23-24**. This biosensor includes the protease recognition site for caspase-8 (**SEQ ID NO:74**) (**Figure 29B**). A similar biosensor utilizes the protease recognition site for caspase-3. (**SEQ ID NO:25-26**)

These biosensors could be used with other biosensors that possess the same product signal color that are targeted to separate compartments, such as CP3GFPNLS-CYTO. The products of each biosensor reaction can be uniquely measured due to separation of the products based on the product targeting sequences. Both products from CP8GFPNUC-CYTO and CP3GFPNLS-CYTO are separable due to the different spatial positions, nucleus vs. nucleolus, even though the colors of the products are exactly the same. Assessing the non-nucleolar, nuclear region in order to avoid the spatial overlap of the two signals would perform the measurement of CP3GFPNLS in

the presence of CP8GFPNUC. The loss of the nucleolar region from the nuclear signal is insignificant and does not significantly affect the SNR. The principle of assessing multiple parameters using the same product color significantly expands the number of parameters that can be assessed simultaneously in living cells. This concept can be extended to other non-overlapping product target compartments.

Measurement of translocation to the nucleolar compartment is performed by (1) defining a mask corresponding to the nucleolus based on a nucleolus-specific marker, including but not limited to an antibody to nucleolin (Lischwe et al., 1981. *Exp. Cell Res.* 136:101-109); (2) defining a mask for the reactant target compartment, and (3) determining the relative distribution of the signal between these two compartments. This relative distribution could be represented by the difference in the two intensities or, preferably, the ratio of the intensities between compartments.

The combination of multiple positional biosensors can be complicated if the reactant compartments are overlapping. Although each signal could be measured by simply determining the amount of signal in each product target compartment, higher SNR will be possible if each reactant is uniquely identified and quantitated. This higher SNR can be maximized by adding a second signal domain of contrasting fluorescent property. This second signal may be produced by a signal domain operatively linked to the product targeting sequence, or by FRET (see above), or by a reactant targeting sequence uniquely identifying it within the reactant compartment based on color, spatial position, or fluorescent property including but not limited to polarization or lifetime. Alternatively, for large compartments, such as the cytoplasm, it is possible to place different, same colored biosensors in different parts of the same compartment.

4. Protease biosensors with multiple copies of a second signal domain serving as a reactant target domain

In another example, (CP8YFPNLS-SIZECFPn) increasing the size of the reactant is accomplished by using multiple inserts of a second signal sequence, for example, ECFP (SEQ ID NO:50) (Figure 29A) (Tsien, R.Y. 1998. *Annu Rev Biochem.* 67:509-44). Thus, the multiple copies of the second signal sequence serve as the reactant target domain by excluding the ability of the biosensor to diffuse into the nucleus. This type of biosensor provides the added benefit of additional signal being

available per biosensor molecule. Aggregation of multiple fluorescent probes also can result in unique signals being manifested, such as FRET, self quenching, excimer formation, etc. This could provide a unique signal to the reactants.

5. Tetanus/botulinum biosensor with trans-membrane targeting domain

In an alternative embodiment, a trans-membrane targeting sequence is used to tether the reactant to cytoplasmic vesicles, and an alternative protease recognition site is used. The tetanus/botulinum biosensor (SEQ ID NOS:27-28 (cellubrevin); 29-30 (synaptobrevin) consists of an NLS (SEQ ID NO:128) (Figure 29C), Fret25' signal domain (SEQ ID NO:52) (Figure 29A), a tetanus or botulinum zinc metalloprotease recognition site from cellubrevin (SEQ ID NO:106) (Figure 29B) (McMahon et al., Nature 364:346-349, 1993; Martin et al., J. Cell Biol., in press) or synaptobrevin (SEQ ID NO:108) (Figure 29B) (GenBank Accession #U64520), and a trans-membrane sequence from cellubrevin (SEQ ID NO:146) (Figure 29C) or synaptobrevin (SEQ ID NO:144) (Figure 29C) at the 3'-end which tethers the biosensor to cellular vesicles. The N-terminus of each protein is oriented towards the cytoplasm. In the intact biosensor, GFP is tethered to the vesicles. Upon cleavage by the tetanus or botulinum zinc metalloprotease, GFP will no longer be associated with the vesicle and is free to diffuse throughout the cytoplasm and the nucleus.

b. 5-domain biosensors

1. Caspase 3 biosensor with a nuclear localization domain and a second signal domain operatively linked to an annexin II domain

The design of this biosensor is outlined in Figure 35, and the sequence is shown in SEQ ID NO:33-34. This biosensor differs from SEQ ID NO 11-12 by including a second detectable signal, ECFP (SEQ ID NO:50) (Figure 29A) (signal 2) operatively linked to the reactant target sequence.

2. Caspase 3 biosensor with a nuclear localization sequence and a second signal domain operatively linked to a MAP4 projection domain (CP3YFPNLS-CFPCYTO)

In this biosensor (SEQ ID NO:31-32), an NLS product targeting domain (SEQ ID NO:128) (Figure 29C) is present upstream of an EYFP signal domain (SEQ ID NO:44) (Figure 29A). A DEVD protease recognition domain (SEQ ID NO:60) (Figure 29B) is between after the EYFP signal domain and before the MAP4 projection domain (SEQ ID NO:142) (Figure 29C).

Example 11. Fluorescent Biosensor Toxin Characterization

As used herein, "toxin" refers to any organism, macromolecule, or organic or inorganic molecule or ion that alters normal physiological processes found within a cell, or any organism, macromolecule, or organic or inorganic molecule or ion that alters the physiological response to modulators of known physiological processes. Thus, a toxin can mimic a normal cell stimulus, or can alter a response to a normal cell stimulus.

Living cells are the targets of toxic agents that can comprise organisms, macromolecules, or organic or inorganic molecules. A cell-based approach to toxin detection, classification, and identification would exploit the sensitive and specific molecular detection and amplification system developed by cells to sense minute changes in their external milieu. By combining the evolved sensing capability of cells with the luminescent reporter molecules and assays described herein, intracellular molecular and chemical events caused by toxic agents can be converted into detectable spatial and temporal luminescent signals.

When a toxin interacts with a cell, whether it is at the cell surface or within a specific intracellular compartment, the toxin invariably undermines one or more components of the molecular pathways active within the cell. Because the cell is comprised of complex networks of interconnected molecular pathways, the effects of a toxin will likely be transmitted throughout many cellular pathways. Therefore, our strategy is to use molecular markers within key pathways likely to be affected by toxins, including but not limited to cell stress pathways, metabolic pathways, signaling pathways, and growth and division pathways.

We have developed and characterized three classes of cell based luminescent reporter molecules to serve as reporters of toxic threat agents. These 3 classes are as follows:

(1) *Detectors*: general cell stress detection of a toxin;

(2) *Classifiers*: perturbation of key molecular pathway(s) for detection and classification of a toxin; and

(3) *Identifiers*: activity mediated detection and identification of a toxin or a group of toxins.

Thus, in another aspect of the present invention, living cells are used as biosensors to interrogate the environment for the presence of toxic agents. In one embodiment of this aspect, an automated method for cell based toxin characterization is disclosed that comprises providing an array of locations containing cells to be treated with a test substance, wherein the cells possess at least a first luminescent reporter molecule comprising a detector and a second luminescent reporter molecule selected from the group consisting of a classifier or an identifier; contacting the cells with the test substance either before or after possession of the first and second luminescent reporter molecules by the cells; imaging or scanning multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the detector; converting the luminescent signals from the detector into digital data to automatically measure changes in the localization, distribution, or activity of the detector on or in the cell, which indicates the presence of a toxin in the test substance; selectively imaging or scanning the locations containing cells that were contacted with test sample indicated to have a toxin in it to obtain luminescent signals from the second reporter molecule; converting the luminescent signals from the second luminescent reporter molecule into digital data to automatically measure changes in the localization, distribution, or activity of the classifier or identifier on or in the cell, wherein a change in the localization, distribution, structure or activity of the classifier identifies a cell pathway that is perturbed by the toxin present in the test substance, or wherein a change in the localization, distribution, structure or activity of the identifier identifies the specific toxin that is present in the test substance. In a preferred embodiment, the cells possess at least a detector, a classifier, and an identifier. In a further preferred embodiment, the digital data derived from the classifier is used to determine which identifier(s) to employ for identifying the specific toxin or group of toxins.

As used herein, the phrase "the cells possess one or more luminescent reporter molecules" means that the luminescent reporter molecule may be expressed as a

luminescent reporter molecule by the cells, added to the cells as a luminescent reporter molecule, or luminescently labeled by contacting the cell with a luminescently labeled molecule that binds to the reporter molecule, such as a dye or antibody, that binds to the reporter molecule. The luminescent reporter molecule can be expressed or added to the cell either before or after treatment with the test substance.

The luminescent reporters comprising detectors, classifiers, and identifiers may also be distributed separately into single or multiple cell types. For example, one cell type may contain a toxin detector, which, when activated by toxic activity, implies to the user that the same toxin sample should be screened with reporters of the classifier or identifier type in yet another population of cells identical to or different from the cells containing the toxin detector.

The detector, classifier, and identifier can comprise the same reporter molecule, or they can comprise different reporters.

Screening for changes in the localization, distribution, structure or activity of the detectors, classifiers, and/or identifiers can be carried out in either a high throughput or a high content mode. In general, a high-content assay can be converted to a high-throughput assay if the spatial information rendered by the high-content assay can be recoded in such a way as to no longer require optical spatial resolution on the cellular or subcellular levels. For example, a high-content assay for microtubule reorganization can be carried out by optically resolving luminescently labeled cellular microtubules and measuring their morphology (e.g., bundled vs. non-bundled or normal). A high-throughput version of a microtubule reorganization assay would involve only a measurement of total microtubule polymer mass after cellular extraction with a detergent. That is, destabilized microtubules, being more easily extracted, would result in a lower total microtubule mass luminescence signal than unperturbed or drug-stabilized luminescently labeled microtubules in another treated cell population. The luminescent signal emanating from a domain containing one or more cells will therefore be proportional to the total microtubule mass remaining in the cells after toxin treatment and detergent extraction.

The methods for detecting, classifying, and identifying toxins can utilize the same screening methods described throughout the instant application, including but not limited to detecting changes in cytoplasm to nucleus translocation, nucleus or nucleolus

to cytoplasm translocation, receptor internalization, mitochondrial membrane potential, signal intensity, the spectral response of the reporter molecule, phosphorylation, intracellular free ion concentration, cell size, cell shape, cytoskeleton organization, metabolic processes, cell motility, cell substrate attachment, cell cycle events, and organellar structure and function.

In all of these embodiments, the methods can be operated in both toxin-mimetic and toxin-inhibitory modes.

Such techniques to assess the presence of toxins are useful for methods including, but not limited to, monitoring the presence of environmental toxins in test samples and for toxins utilized in chemical and biological weapons; and for detecting the presence and characteristics of toxins during environmental remediation, drug discovery, clinical applications, and during the normal development and manufacturing process by virtually any type of industry, including but not limited to agriculture, food processing, automobile, electronic, textile, medical device, and petroleum industries.

We have developed and characterized examples of luminescent cell-based reporters, distributed across the 3 sensor classes. The methods disclosed herein can be utilized in conjunction with computer databases, and data management, mining, retrieval, and display methods to extract meaningful patterns from the enormous data set generated by each individual reporter or a combinatorial of reporters in response to toxic agents. Such databases and bioinformatics methods include, but are not limited to, those disclosed in U.S. Patent Application Nos. 09/437,976, filed November 10, 1999; 60/145,770 filed July 27, 1999 and U.S. Patent Application Serial No. to be assigned, filed February 19, 2000. (98,068-C)

Any cell type can be used to carry out this aspect of the invention, including prokaryotes such as bacteria and archaeobacteria, and eukaryotes, such as single celled fungi (for example, yeast), molds (for example, Dictyostelium), and protozoa (for example, Euglena). Higher eukaryotes, including, but not limited to, avian, amphibian, insect, and mammalian cells can also be used.

Examples of Biosensors

Number	Name	Class	Cell Types	Response to model toxins
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				Positive	Negative
1	Mitochondrial Potential [Donnan Equilibrium Dye]	D	<ul style="list-style-type: none"> • LLCPK (pig epithelia) • Rat primary hepatocytes 	Valinomycin (10 nM-100 μ M) FCCP (10 nM-100 μ M)	Oligomycin (10 nM)
2	Heat Shock Protein (Hsp 27, Hsp 70) GFP-chimera	D	<ul style="list-style-type: none"> • HeLa • 3T3 	Cadmium (10mM)	TNF- α (100ng/ml)
3	Tubulin-cytoskeleton [β -tubulin-GFP chimera]	C	<ul style="list-style-type: none"> • BHK • HeLa • LLCPK 	Paclitaxel (10 nM-20 μ M) Curacin-A (5 nM-10 μ M) Nocadazole (7 nM-12 μ M) Colchicine (5 nM-10 μ M) Vinblastine (5 nM-10 μ M)	Staurosporine (1 nM-1 μ M)
4	pp38 MAPK- stress signaling [antibody and GFP-chimera]	C	<ul style="list-style-type: none"> • 3T3 • LLCPK 	Anisomycin (100 μ M) Cadmium (10 μ M)	TNF- α (100 ng/ml)
5	NF- κ B- stress signaling [antibody and GFP-chimera]	C	<ul style="list-style-type: none"> • HeLa • 3T3 • BHK • SNB19 • HepG2 • LLCPK 	TNF- α (100ng/ml-0.38pg/ml) IL-1 (4ng/ml-.095pg/ml) Nisin (2-1000 μ g/ml) Streptolysin (10 μ g/ml) Anisomycin (100 μ M)	Anisomycin (10 nM-10 μ M) Cadmium (1-10 μ M) Penitrem A (10 μ M) Valinomycin (1 μ M)
6	I κ B [complement to NF- κ B]	C	In many cell types		
7	Tetanus Toxin [Protease activity-based sensor]	I	In many cell types		
8	Anthrax LF [Protease activity-based sensor]	I	In many cell types		

Sensor Class: D= Detector of toxins; C= Classifier of toxins; I= Identifier of toxin or group of toxins

The model toxins can generally be purchased from Sigma Chemical Company (St. Louis, MO)

- 5 **Examples of Detectors:** This class of sensors provides a first line signal that indicates the presence of a toxic agent. This class of sensors provides detection of general cellular stress that requires resolution limited only to the domain over which the measurement is being made, and they are amenable to high content screens as well. Thus, either high throughput or high content screening modes may be used, including
- 10 but not limited to translocation of heat shock factors from the cytoplasm to the nucleus,

and changes in mitochondrial membrane potential, intracellular free ion concentration detection (for example, Ca^{2+} ; H^+), general metabolic status, cell cycle timing events, and organellar structure and function.

5 1. Mitochondrial Potential

A key to maintenance of cellular homeostasis is a constant ATP energy charge. The cycling of ATP and its metabolites ADP, AMP, inorganic phosphate, and solution-phase protons is continuously adjusted to meet the catabolic and anabolic needs of the cell. Mitochondria are primarily responsible for maintaining a constant energy charge
10 throughout the entire cell. To produce ATP from its constituents, mitochondria must maintain a constant membrane potential within the organelle itself. Therefore, measurement of this electrical potential with specific luminescent probes provides a sensitive and rapid readout of cellular stress.

We have utilized a positively charged cyanine dye, JC-1 (Molecular Probes,
15 Eugene, OR), which diffuses into the cell and readily partitions into the mitochondrial membrane, for measurement of mitochondrial potential. The photophysics of JC-1 are such that when the probe partitions into the mitochondrial membrane and it experiences an electrical potential >140 mV, the probe aggregates and its spectral response is shifted to the red. At membrane potential values <140 mV, JC-1 is primarily
20 monomeric and its spectral response is shifted toward the blue. Therefore, the ratio of two emission wavelengths (645 nm and 530 nm) of JC-1 partitioned into mitochondria provides a sensitive and continuous measure of mitochondrial membrane potential.

We have been making live cell measurements in a high throughput mode as the basis of a generalized indicator of toxic stress. The goal of our initial experiments was
25 to determine the ratio of J-aggregates of JC-1 dye to its monomeric form both before and after toxic stress.

Procedure

1. Cells were plated and cultured up to overnight.
2. Cells were stained with JC-1 (10 $\mu\text{g}/\text{ml}$) for 30 minutes at 37°C in a CO_2 incubator.
- 30 3. Cells were then washed quickly with HBSS at 37°C (2 times, 150 $\mu\text{l}/\text{well}$), the toxins were added if required, and the entire plate was scanned in a plate reader. The JC-1 monomer was measured optimally with a 485 nm excitation/530 nm emission wavelength filter set, and the aggregates were best measured with a 590 nm excitation/645 nm emission wavelength set.

Results

The mitochondrial potential within several types of living cells, and the effects of toxins on the potential were measured using the fluorescence ratio Em 645 (590)/Em 530 (485) (excitation wavelengths in parentheses). For example, we measured the effect of 10 μ M valinomycin on the mitochondrial potential within LLCPK cells (pig epithelia). Within seconds of treatment, the toxin induced a more rapid and higher magnitude decrease (an approximately 50% reduction) in mitochondrial potential than that found in untreated cells. Hepatocytes were also determined to be sensitive to valinomycin, and the changes in mitochondrial potential were nearly complete within seconds to minutes after addition of various concentrations of the toxin.

These results are consistent with mitochondrial potential being a model intracellular detector of cell stress. Because these measurements require no spatial resolution within individual cells, mitochondrial potential measurements can be made rapidly on an entire cell array (e.g. high throughput). This means, for example, that complex arrays of many cell types can be probed simultaneously and continuously as a generalized toxic response. Such an indicator can provide a first line signal to indicate that a general toxic stress is present in a sample. Further assays can then be conducted to more specifically identify the toxin using cells classifier or identifier type reporter molecules.

2. Heat Shock Proteins

Most mammalian cells will respond to a variety of environmental stimuli with the induction of a family of proteins called stress proteins. Anoxia, amino acid analogues, sulfhydryl-reacting reagents, transition metal ions, decouplers of oxidative phosphorylation, viral infections, ethanol, antibiotics, ionophores, non-steroidal antiinflammatory drugs, thermal stress and metal chelators are all inducers of cell stress protein synthesis, function, or both. Upon induction, cell stress proteins play a role in folding and unfolding proteins, stabilizing proteins in abnormal configurations, and repairing DNA damage.

There is evidence that at least four heat shock proteins translocate from the cytoplasm to the nucleus upon stress activation of the cell. These proteins include the

heat shock proteins HSP27 and HSP70, the heat shock cognate HSC70, and the heat shock transcription factor HSF1. Therefore, measurement of cytoplasm to nuclear translocation of these proteins (and other stress proteins that translocate from the cytoplasm to the nucleus upon a cell stress) will provide a rapid readout of cellular stress.

We have tested the response of an HSP27-GFP biosensor (SEQ ID 169-170) in two cell lines (BHK and HeLa) using a library of heavy metal chemical compounds as biological toxin stimulants to stress the cells. Briefly, cells expressing the HSP27-GFP biosensor are plated into 96-well microplates, and allowed to attach. The cells are then treated with a panel of cell stress-inducing compounds. Exclusively cytoplasmic localization of the fusion protein was found in unstimulated cells.

Other similar heat shock protein biosensors (HSP-70, HSC70, and HSF1 fused to GFP) can be used as detectors, and are shown in SEQ ID NO: 171-176.

Examples of Classifiers:

This class of sensors detects the presence of, and further classifies toxins by identifying the cellular pathway(s) perturbed by the toxin. As such, this suite of sensors can detect and/or classify toxins into broad categories, including but not limited to “toxins affecting signal transduction,” “toxins affecting the cytoskeleton,” and “toxins affecting protein synthesis”. Either high throughput or high content screening modes may be used. Classifiers can comprise compounds including but not limited to tubulin, microtubule-associated proteins, actin, actin-binding proteins including but not limited to vinculin, α -actinin, actin depolymerizing factor/cofilin, profilin, and myosin; NF- κ B, I κ B, GTP-binding proteins including but not limited to rac, rho, and cdc42, and stress-activated protein kinases including but not limited to p38 mitogen-activated protein kinase.

1. Tubulin-cytoskeleton

The cell cytoskeleton plays a major role in cellular functions and processes, such as endo- and exocytosis, vesicle transport, and mitosis. Cytoskeleton-affecting

toxins, of proteinaceous and non-proteinaceous form, such as C2 toxin, and several classes of enterotoxins, act either directly on the cytoskeleton, or indirectly via regulatory components controlling the organization of the cytoskeleton. Therefore, measurement of structural changes in the cytoskeleton can provide classification of the toxin into a class of cytoskeleton-affecting toxins. This assay can be conducted in a high content mode, as described previously, or in a high throughput mode. For high throughput as discussed previously.

Such measurements will be valuable for identification of toxins including, but not limited to anti-microtubule agents, agents that generally affect cell cycle progression and cell proliferation, intracellular signal transduction, and metabolic processes.

For microtubule disruption assays, LLCPK cells stably transfected with a tubulin-GFP biosensor plasmid were plated on 96 well cell culture dishes at 50-60% confluence and cultured overnight at 37 °C, 5% CO₂. A series of concentrations (10–500 nM) of 5 compounds (paclitaxel, curacin A, nocodazole, vinblastine, and colchicine) in normal culture media were freshly prepared from stock, and were added to cell culture dishes to replace the old culture media. The cells were then observed with the cell screening system described above, at a 12 hour time point.

Our data indicate that the tubulin chimera localizes to and assembles into microtubules throughout the cell. The microtubule arrays in cells expressing the chimera respond as follows to a variety of anti-microtubule compounds:

<u>Drug</u>	<u>Response</u>
Vinblastine	Destabilization
Nocodazole	Destabilization
Paclitaxel	Stabilization
Colchicine	Destabilization
Curacin A	Destabilization

Similar data were obtained using cells expressing the tubulin biosensor that were patterned onto cell arrays (such as those described in U.S. Patent Application Serial No. 08/865,341 filed May 29, 1997, incorporated by reference herein in its entirety) and dosed as above.

2. NF- κ B

NF- κ B is cytoplasmic at basal levels of stimulation, but upon insult translocates to the nucleus where it binds specific DNA response elements and activates transcription of a number of genes. Translocation occurs when I κ B is degraded by the proteasome in response to specific phosphorylation and ubiquitination events. I κ B normally retains NF- κ B in the cytoplasm via direct interaction with the protein, and masking of the NLS sequence of NF- κ B. Therefore, although not the initial or defining event of the whole signal cascade, NF- κ B translocation to the nucleus can serve as an indicator of cell stress.

We have generated an NF- κ B-GFP chimera for analysis in live cells. This was accomplished using standard polymerase chain reaction techniques using a characterized NF- κ B p65 cDNA purchased from Invitrogen (Carlsbad, CA) fused to an EYFP PCR amplimer that was obtained from Clontech Laboratories (Palo Alto, CA). The resulting chimera is shown in SEQ ID NO:177-178. The two PCR products were ligated into an eukaryotic expression vector designed to produce the chimeric protein at high levels using the ubiquitous CMV promoter.

NF- κ B immunolocalization

For further studies, we characterized endogenous NF- κ B activation by immunolocalization in toxin treated cells. The NF- κ B antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and secondary antibodies are from Molecular Probes (Eugene, OR).

For the 3T3 and SNB19 cell types, we determined the effective concentrations that yield response levels of 50% of the maximum (EC50), expressed in units of mass per volume (ng/ml) and units of molarity. Based on molecular weights of 17 kD for both TNF α and IL-1 α , the EC50 levels for these two compounds with 3T3 and SNB19 cell types are given in units of molarity in Table 1. Our results demonstrated reproducibility of the relative responses from zero to maximum dose, but from sample to sample there have been occasional shifts in the baseline intensities of the response at zero concentration.

For these experiments, either 10 or 100 TNF α -treated 3T3 or SNB19 cells/well were tested. On the basis of the standard deviations measured for these samples, and by taking t-values for the student's t-test, we have estimated the minimum detectable doses for each case of cell type, compound, number of cells per well, and for different choices of how many wells are sampled per condition. The latter factor determines the number of degrees of freedom that are provided in the sample of data. Increasing the number of wells from 4 to 16, and increasing the number of cells per well from 10 to 100, improves the minimum detectable doses considerably. For 3T3 cells, which show lower minimum detectable doses than the SNB19 cells, and for the case of 1% false negative and 1% false positive rates, we estimate that 100 cells per well and a sampling of 12 or 16 wells are sufficient to detect a dose approximately equal to the EC50 value of 0.15 ng/ml. If the false positive rate is relaxed to 20%, a concentration of approximately half that value can be detected (0.83 ng/ml). One hundred cells can conveniently be sampled over a cell culture surface area of less than 1 mm².

Table 1. EC50 levels for TNF α and IL-1 α (based on molecular weights of 17 kD for both)

Compound	Cell Type	EC50 (10 ⁻¹² moles/liter)
TNF α	3T3	8.8
	SNB19	5.9
IL-1 α	3T3	0.24
	SNB19	59

3. *Phospho-p38 Mitogen Activated Protein Kinase (pp38MAPK)*

MAPKs play a role in not only cell growth and division, but as mediators of cellular stress responses. One MAPK, p38, is activated by chemical stress inducers such as hyper-osmolar sorbitol, hydrogen peroxide, arsenite, cadmium ions, anisomycin, sodium salicylate, and LPS. Activation of p38 is also accompanied by its translocation into the nucleus from the cytoplasm.

MAPK p38 lies in a pathway that is a cascade of kinases. Thus, p38 is a substrate of one or more kinases, and it acts to phosphorylate one or more substrates in time and space within the living cell.

The assay we present here measures, as one of its parameters, p38 activation using immunolocalization of the phosphorylated form of p38 in toxin-treated cells. The assay was developed to be flexible enough to include the simultaneous measurement of other parameters within the same individual cells. Because the signal transduction pathway mediated by the transcription factor NF- κ B is also known to be involved in the cell stress response, we included the activation of NF- κ B as a second parameter in the same assay.

Our experiments demonstrate an immunofluorescence approach can be used to measure p38 MAPK activation either alone or in combination with NF- κ B activation in the same cells. Multiple cell types, model toxins, and antibodies were tested, and significant stimulation of both pathways was measured in a high-content mode. The phospho-p38 antibodies used in this study were purchased from Sigma Chemical Company (St. Louis, MO). We report that at least two cell stress signaling pathways can not only be measured simultaneously, but are differentially responsive to classes of model toxins. **Figure 36** shows the differential response of the p38 MAPK and NF- κ B pathways across three model toxins and two different cell types. Note that when added alone, three of the model toxins (IL1 α , TNF α and Anisomycin) can be differentiated by the two assays as activators of specific pathways.

I κ B chimera

I κ B degradation is the key event leading to nuclear translocation of NF- κ B and activation of the NF κ B-mediated stress response. We have chosen this sensor to complement the NF- κ B sensor as a *classifier* in a high-throughput mode: the measurement of loss of signal due to degradation of the I κ B-GFP fusion protein requires no spatial resolution within individual cells, and as such we envision I κ B degradation measurements being made rapidly on an entire cell substrate.

This biosensor is based on fusion of the first 60 amino acids of I κ B to the Fred25 variant of GFP. **SEQ ID 179-180** This region of I κ B contains all the regulatory

sequences, including phosphorylation sites and ubiquitination sites, necessary to confer proteasome degradation upon the biosensor. Knowing this, stimulation of any pathway that would typically lead to NFkB translocation results in degradation of this biosensor. Monitoring the fluorescence intensity of cells expressing Ikb-GFP identifies the degradation process.

Examples of Identifiers:

In our toxin identification strategy, the first two levels of characterization ensure a rapid readout of toxin class without sacrificing the ability to detect many new mutant toxins or dissect several complex mixtures of known toxins. The third level of biosensors are identifiers, which can identify a specific toxin or group of toxins. In one embodiment, an identifier comprises a protease biosensor that responds to the activity of a specific toxin. Other identifiers are produced with reporters/biosensors specific to their activities. These include, but are not limited to post-translational modifications such as phosphorylation or ADP-ribosylation, translocation between cellular organelles or compartments, effects on specific organelles or cellular components (for example, membrane permeabilization, cytoskeleton rearrangement, etc.)

ADP-ribosylating toxins – These toxins include Pseudomonas toxin A, diphtheria toxin, botulinum toxin, pertussis toxin, and cholera toxin. For example, C. botulinum C2 toxin induces the ADP-ribosylation of Arg177 in the cytoskeletal protein actin, thus altering its assembly properties. Besides the construction of a classifier assay to measure actin-cytoskeleton regulation, an identifier assay can be constructed to detect the specific actin ADP-ribosylation. Because the ADP-ribosylation induces a conformational change that no longer permits the modified actin to polymerize, this conformational change can be detected intracellularly in several possible ways using luminescent reagents. For example, actin can be luminescently labeled using a fluorescent reagent with an appropriate excited state lifetime that allows for the measurement of the rotational diffusion of the intracellular actin using steady state fluorescence anisotropy. That is, toxin-modified actin will no longer be able to assemble into rigid filaments and will therefore produce only luminescent signals with

relatively low anisotropy, which can be readily measured with an imaging system. In another embodiment, actin can be labeled with a polarity-sensitive fluorescent reagent that reports changes in actin-conformation through spectral shifts of the attached reagent. That is, toxin-treatment will induce a conformational change in intracellular actin such that a ratio of two fluorescence wavelengths will provide a measure of actin ADP-ribosylation.

Cytotoxic phospholipases – Several gram-positive bacterial species produce cytotoxic phospholipases. For example, *Clostridium perfringens* produces a phospholipase C specific for the cleavage of phosphoinositides. These phosphoinositides (e.g., inositol 1,4,5-trisphosphate) induce the release of calcium ions from intracellular organelles. An assay that can be conducted as either high-content or high-throughput can be constructed to measure the release of calcium ions using fluorescent reagents that have altered spectral properties when complexed with the metal ion. Therefore, a direct consequence of the action of a phospholipase C based toxin can be measured as a change in cellular calcium ion concentration.

Exfoliative toxins – These toxins are produced by several *Staphylococcal* species and can consist of several serotypes. A specific identifier for these toxins can be constructed by measuring the morphological changes in their target organelle, the desmosome, which occur at the junctions between cells. The exfoliative toxins are known to change the morphology of the desmosomes into two smaller components called hemidesmosomes. In the high-content assay for exfoliative toxins, epithelial cells whose desmosomes are luminescently labeled are subjected to image analysis. An method that detects the morphological change between desmosomes and hemidesmosomes is used to quantify the activity of the toxins on the cells.

Most of these identifiers can be used in high throughput assays requiring no spatial resolution, as well as in high content assays.

Several biological threat agents act as specific proteases, and thus we have focused on the development of fluorescent protein biosensors that report the proteolytic cleavage of specific amino acid sequences found within the target proteins.

A number of such protease biosensors (including FRET biosensors) are disclosed above, such as the caspase biosensors, anthrax, tetanus, Botulinum, and the

zinc metalloproteases. FRET is a powerful technique in that small changes in protein conformation, many of which are associated with toxin activity, can not only be measured with high precision in time and space within living cells, but can be measured in a high-throughput mode, as discussed above.

5 As described above, one of skill in the art will recognize that the protease biosensors of this aspect of the invention can be adapted to report the activity of any protease, by a substitution of the appropriate protease recognition site in any of the constructs (see **Figure 29B**). As disclosed above, these biosensors can be used in high-content or high throughput screens to detect in vivo activation of enzymatic activity by
10 toxins, and to identify specific activity based on cleavage of a known recognition motif. These biosensors can be used in both live cell and fixed end-point assays, and can be combined with additional measurements to provide a multi-parameter assay.

Anthrax LF

15 Anthrax is a well-known agent of biological warfare and is an excellent target for development of a biosensor in the *identifier* class. Lethal factor (LF) is one of the protein components that confer toxicity to anthrax, and recently two of its targets within cells were identified. LF is a metalloprotease that specifically cleaves Mek1 and Mek2 proteins, kinases that are part of the MAP-kinase signaling pathway. Construction of
20 lethal factor protease biosensors are described above. (SEQ ID NO:7-8; 9-10) Green fluorescent protein (GFP) is fused in-frame at the amino terminus of either Mek1 or Mek2 (or both), resulting in a chimeric protein that is retained in the cytoplasm due to the presence of a nuclear export sequence (NES) present in both of the target molecules. Upon cleavage by active lethal factor, GFP is released from the chimera and
25 is free to diffuse into the nucleus. Therefore, measuring the accumulation of GFP in the nucleus provides a direct measure of LF activity on its natural target, the living cell.

While a preferred form of the invention has been shown in the drawings and described, since variations in the preferred form will be apparent to those skilled in the art, the invention should not be construed as limited to the specific form shown and
30 described, but instead is as set forth in the claims.

CLAIMS

We claim:

1. An automated method for cell based toxin characterization comprising

-providing an array of locations containing cells to be treated with a test
5 substance, wherein the cells possess at least a first luminescent reporter molecule
comprising a detector and a second luminescent reporter molecule selected from the
group consisting of a classifier or an identifier;

-contacting the cells with the test substance either before or after possession of
the first and second luminescent reporter molecules by the cells; wherein the
10 localization, distribution, structure, or activity of the first and second luminescent
reporter molecule is modified when the cell is contacted with the toxin,

-imaging or scanning multiple cells in each of the locations containing multiple
cells to obtain luminescent signals from the detector;

-converting the luminescent signals from the detector into digital data;

15 -utilizing the digital data from the detector to automatically measure the
localization, distribution, or activity of the detector on or in the cell, wherein a change
in the localization, distribution, structure or activity of the detector indicates the
presence of a toxin in the test substance;

-selectively imaging or scanning the locations containing cells that were
20 contacted with test sample indicated to have a toxin in it to obtain luminescent signals
from the second reporter molecule;

-converting the luminescent signals from the second luminescent reporter
molecule into digital data;

25 -utilizing the digital data from the second luminescent reporter molecule to
automatically measure the localization, distribution, or activity of the classifier or
identifier on or in the cell, wherein a change in the localization, distribution, structure
or activity of the classifier identifies a cell pathway that is perturbed by the toxin
present in the test substance, or wherein a change in the localization, distribution,
structure or activity of the identifier identifies the specific toxin or group of toxins that
30 are present in the test substance.

2. The method of claim 1 wherein the second luminescent reporter molecule is a classifier, and the digital data derived from the classifier is used to select an appropriate identifier for identification of the specific toxin or group of toxins.

5 3. An automated method for cell based toxin characterization comprising

-providing an array of locations containing cells to be treated with a test substance, wherein the cells possess at least a first luminescent reporter molecule comprising a detector, a second luminescent reporter molecule comprising a classifier, and a third luminescent reporter molecule comprising an identifier;

10 -contacting the cells with the test substance either before or after possession of the first second, and third luminescent reporter molecules by the cells; wherein the localization, distribution, structure, or activity of the first, second, and third luminescent reporter molecules is modified when the cell is contacted with the toxin,

-imaging or scanning multiple cells in each of the locations containing multiple
15 cells to obtain luminescent signals from the detector;

-converting the luminescent signals from the detector into digital data;

-utilizing the digital data from the detector to automatically measure the localization, distribution, or activity of the detector on or in the cell, wherein a change in the localization, distribution, structure or activity of the detector indicates the
20 presence of a toxin in the test substance;

-selectively imaging or scanning the locations containing cells that were contacted with test sample indicated to have a toxin in it to obtain luminescent signals from the classifier;

-converting the luminescent signals from the classifier into digital data;

25 -utilizing the digital data from the classifier to automatically measure the localization, distribution, or activity of the classifier on or in the cell, wherein a change in the localization, distribution, structure or activity of the classifier identifies a cell pathway that is perturbed by the toxin present in the test substance;

--selectively imaging or scanning the locations containing cells that were
30 contacted with test sample indicated to have a toxin in it to obtain luminescent signals from the identifier;

-converting the luminescent signals from the identifier into digital data; and

-utilizing the digital data from the identifier to automatically measure the localization, distribution, or activity of the identifier on or in the cell, wherein a change in the localization, distribution, structure or activity of the identifier identifies the specific toxin or group of toxins that is present in the test substance.

5

4. The method of claim 3 wherein the digital data derived from the classifier is used to select an appropriate identifier for identification of the specific toxin or group of toxins.

10

5. The method of any one of claim 1-4 wherein the detector comprises a molecule selected from the group consisting of heat shock proteins and compounds that respond to changes in mitochondrial membrane potential, intracellular free ion concentration, cytoskeletal organization, general metabolic status, cell cycle timing events, and organellar structure and function.

15

6. The method of any one of claim 1-5 wherein the classifier comprises a molecule selected from the group consisting of tubulin, microtubule-associated proteins, actin, actin-binding proteins, NF- κ B, I κ B, and stress-activated kinases.

20

7. The method of any one of claim 1-6 wherein the cell pathway is selected from the group consisting of cell stress pathways, cell metabolic pathways, cell signaling pathways, cell growth pathways, and cell division pathways.

25

8. The method of claim 1, wherein the second luminescent reporter molecule is an identifier, and the identifier identifies a toxin or group of toxins selected from the group consisting of proteases, ADP-ribosylating toxins, cytotoxic phospholipases, and exfoliative toxins.

30

9. The method of any one of claim 3-7, wherein the identifier identifies a toxin or group of toxins selected from the group consisting of proteases, ADP-ribosylating toxins, cytotoxic phospholipases, and exfoliative toxins.

10. The method of any of claims 1-9 wherein the change in the localization, distribution, structure or activity of the first, second, or third luminescent reporter molecules is selected from the group consisting of cytoplasm to nucleus translocation, nucleus or nucleolus to cytoplasm translocation, receptor internalization, mitochondrial membrane potential, loss of signal, the spectral response of the reporter molecule, phosphorylation, intracellular free ion concentration, cell size, cell shape, cytoskeleton organization, metabolic processes, cell motility, cell substrate attachment, cell cycle events, and organellar structure and function.
11. The method of any one of claims 1-10, wherein the imaging or scanning multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the detector is carried out in a high throughput mode.
12. The method of any one of claims 1-10, wherein the imaging or scanning multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the detector is carried out in a high content mode.
13. The method of claim 1-10 wherein the selective imaging or scanning of the locations containing cells that were contacted with test sample indicated to have a toxin in it to obtain luminescent signals from the second or third reporter molecule is carried out in a high throughput mode.
14. The method of claim 1-10 wherein the selective imaging or scanning of the locations containing cells that were contacted with test sample indicated to have a toxin in it to obtain luminescent signals from the second or third reporter molecule is carried out in a high content mode.
15. The method of any one of claims 1-14 further comprising providing a digital storage media for data storage and archiving.
16. The method of claim 15 further comprising a means for automated control, acquisition, processing and display of results.

17. A computer readable storage medium comprising a program containing a set of instructions for causing a cell screening system to execute the method of any one of claims 1-16, wherein the cell screening system comprises an optical system with a stage adapted for holding a plate containing cells, a means for moving the stage or the optical system, a digital camera, a means for directing light emitted from the cells to the digital camera, and a computer means for receiving and processing the digital data from the digital camera.

18. A kit for cell based toxin detection comprising:

(a) at least one reporter molecule, wherein the localization, distribution, structure, or activity of the reporter molecule is modified when the cell is contacted with a toxin;

(b) instructions for using the reporter molecule to carry out the method of any one of claims 1-16 to detect toxins in a test substance.

19. The kit of claim 18 further comprising the computer readable storage medium of claim 17.

20. An automated method for cell based toxin characterization comprising

-providing a first array of locations containing cells to be treated with a test substance, wherein the cells possess a least a first luminescent reporter molecule comprising a reporter molecule selected from the group consisting of detectors and classifiers;

-contacting the cells with the test substance either before or after possession of the first luminescent reporter molecule by the cells; wherein the localization, distribution, structure, or activity of the first luminescent reporter molecule is modified when the cell is contacted with the toxin,

-imaging or scanning multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the detector;

-converting the luminescent signals from the detector into digital data;

-utilizing the digital data from the detector to automatically measure the localization, distribution, or activity of the detector on or in the cell, wherein a change in the localization, distribution, structure or activity of the detector indicates the presence of a toxin in the test substance,

5 -providing a second array of locations containing cells to be treated with the test substance, wherein the cells possess a least a second luminescent reporter molecule comprising a reporter molecule selected from the group consisting of classifiers and identifiers, and wherein the second array of locations containing cells can comprise either the same or a different cell type as the first array of locations containing cells;

10 -contacting the second array of locations containing cells with the test substance either before or after possession of the second luminescent reporter molecule by the cells; wherein the localization, distribution, structure, or activity of the second luminescent reporter molecule is modified when the cell is contacted with the toxin;

15 -utilizing the digital data from the second luminescent reporter molecule to automatically measure the localization, distribution, or activity of the classifier or identifier on or in the cell, wherein a change in the localization, distribution, structure or activity of the classifier identifies a cell pathway that is perturbed by the toxin present in the test substance, or wherein a change in the localization, distribution, structure or activity of the identifier identifies the specific toxin or group of toxins that
20 are present in the test substance.

Figure 1

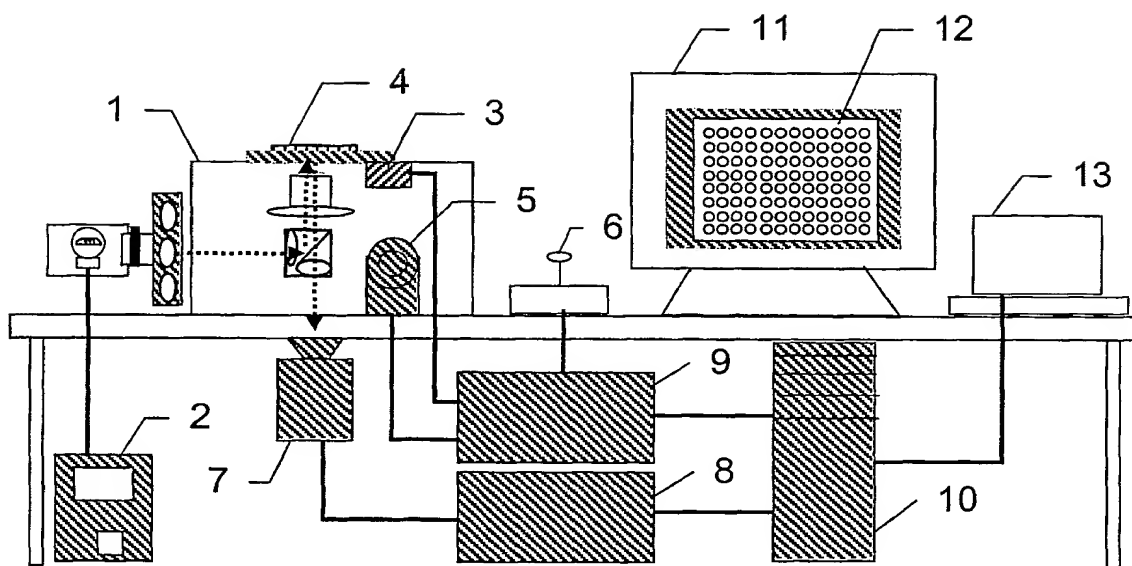


Figure 2

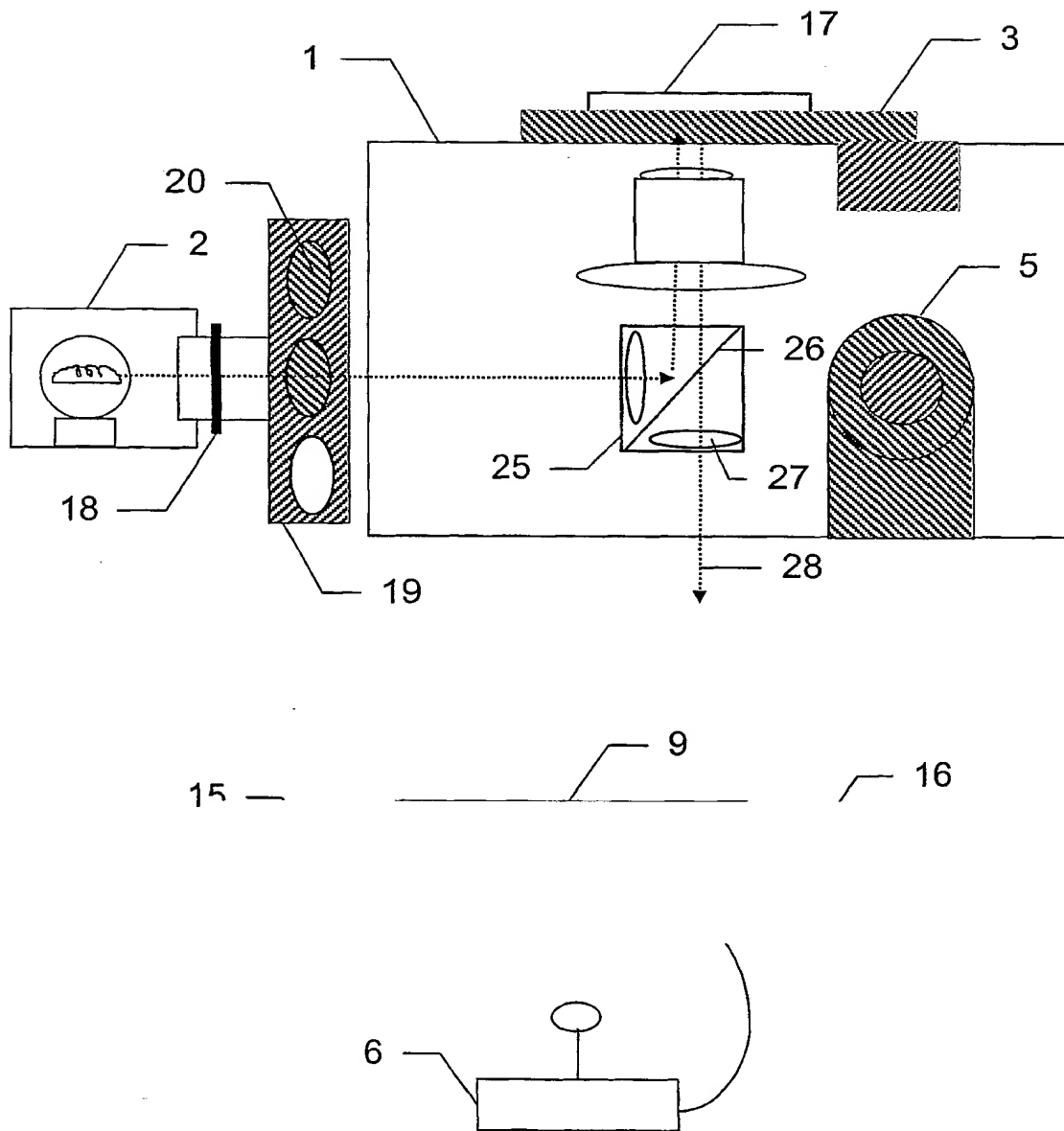


Figure 3

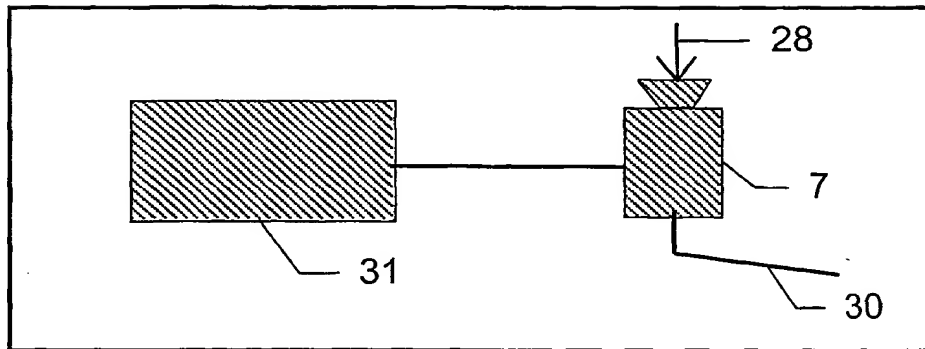


Figure 4

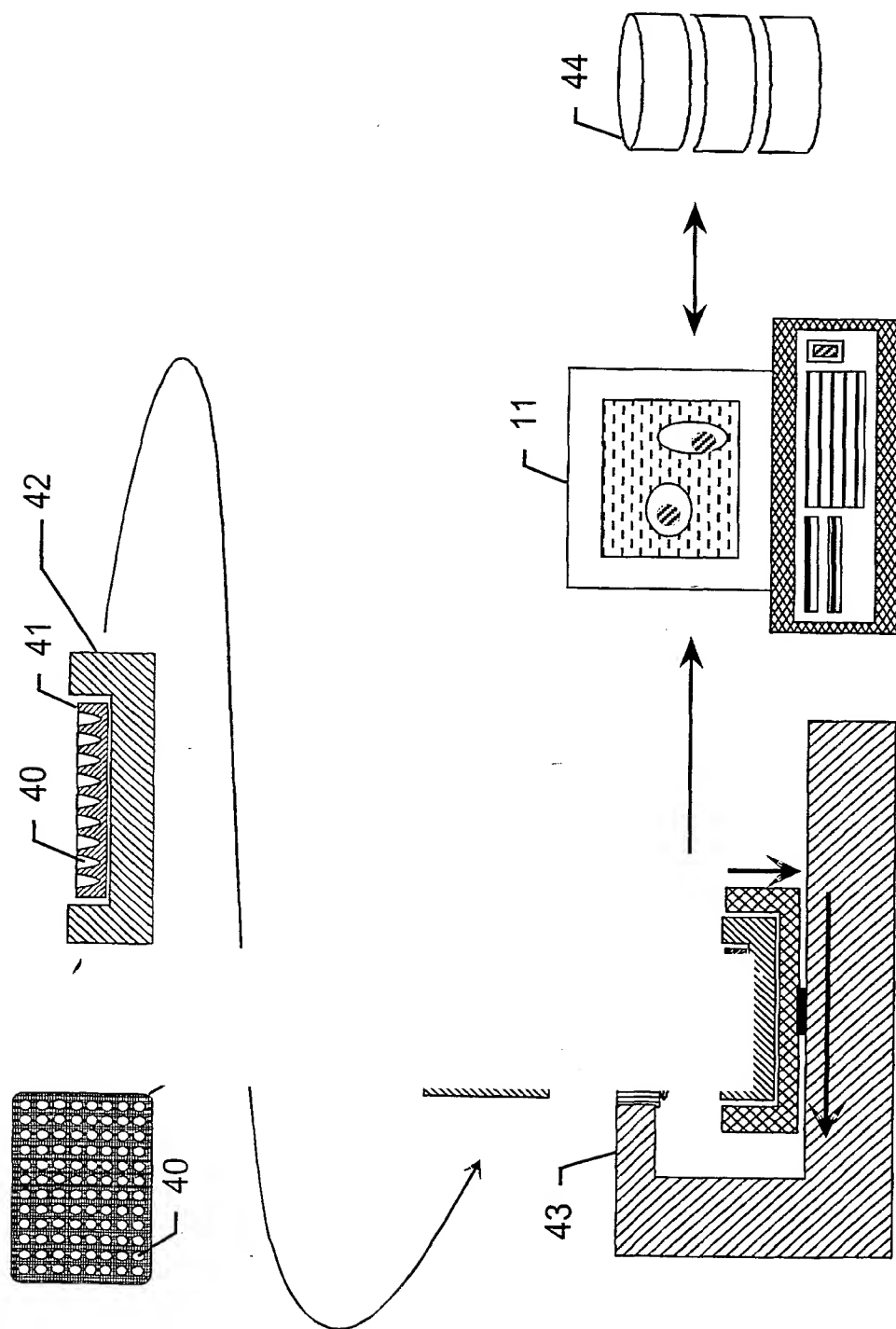


Figure 5

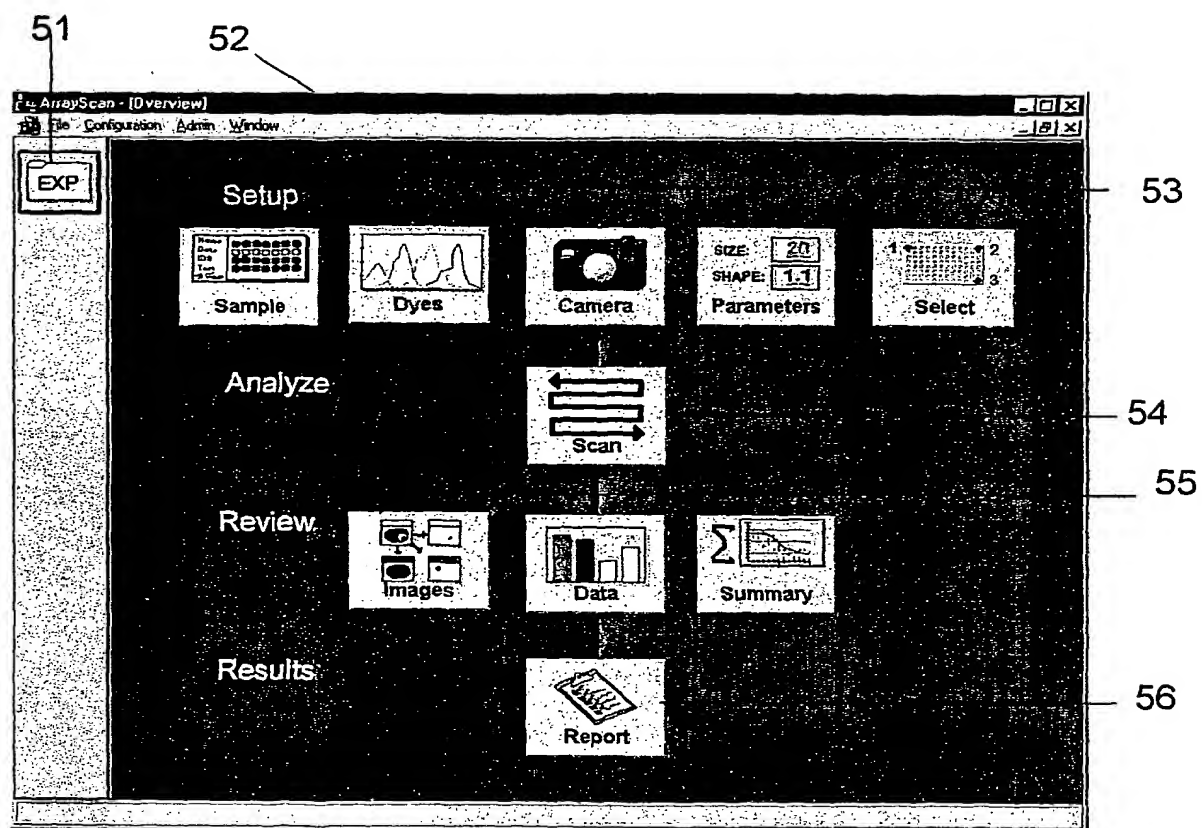


Figure 6

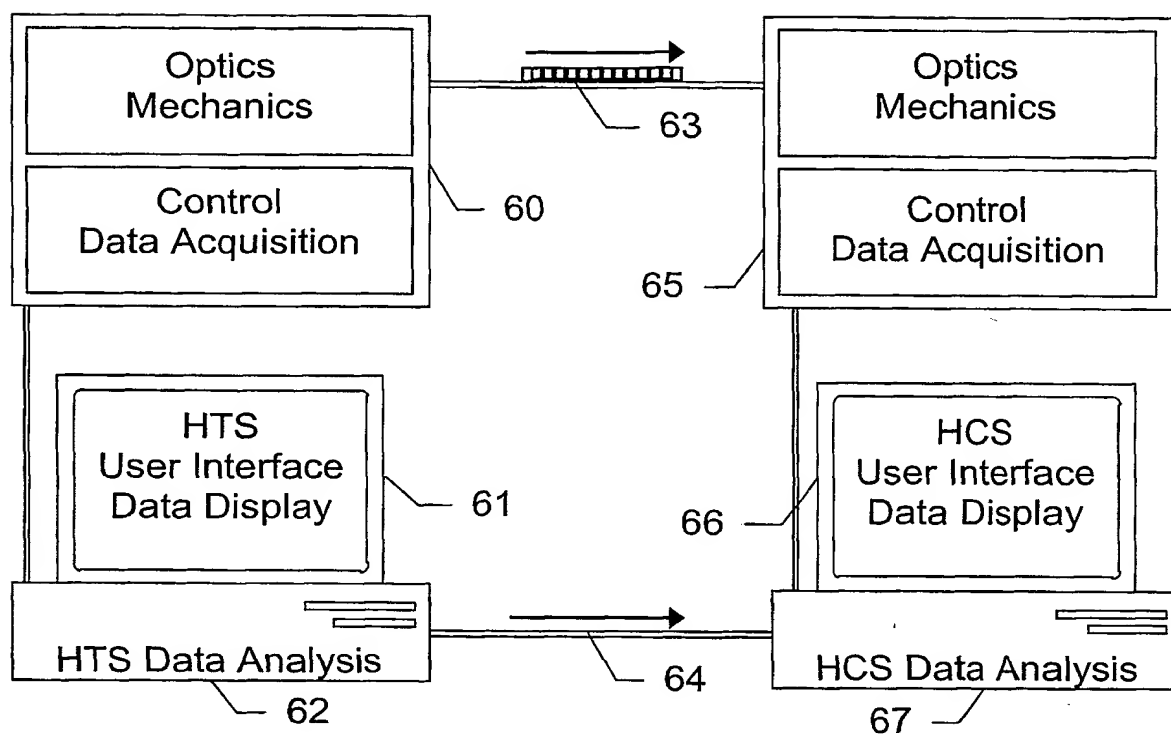


Figure 7

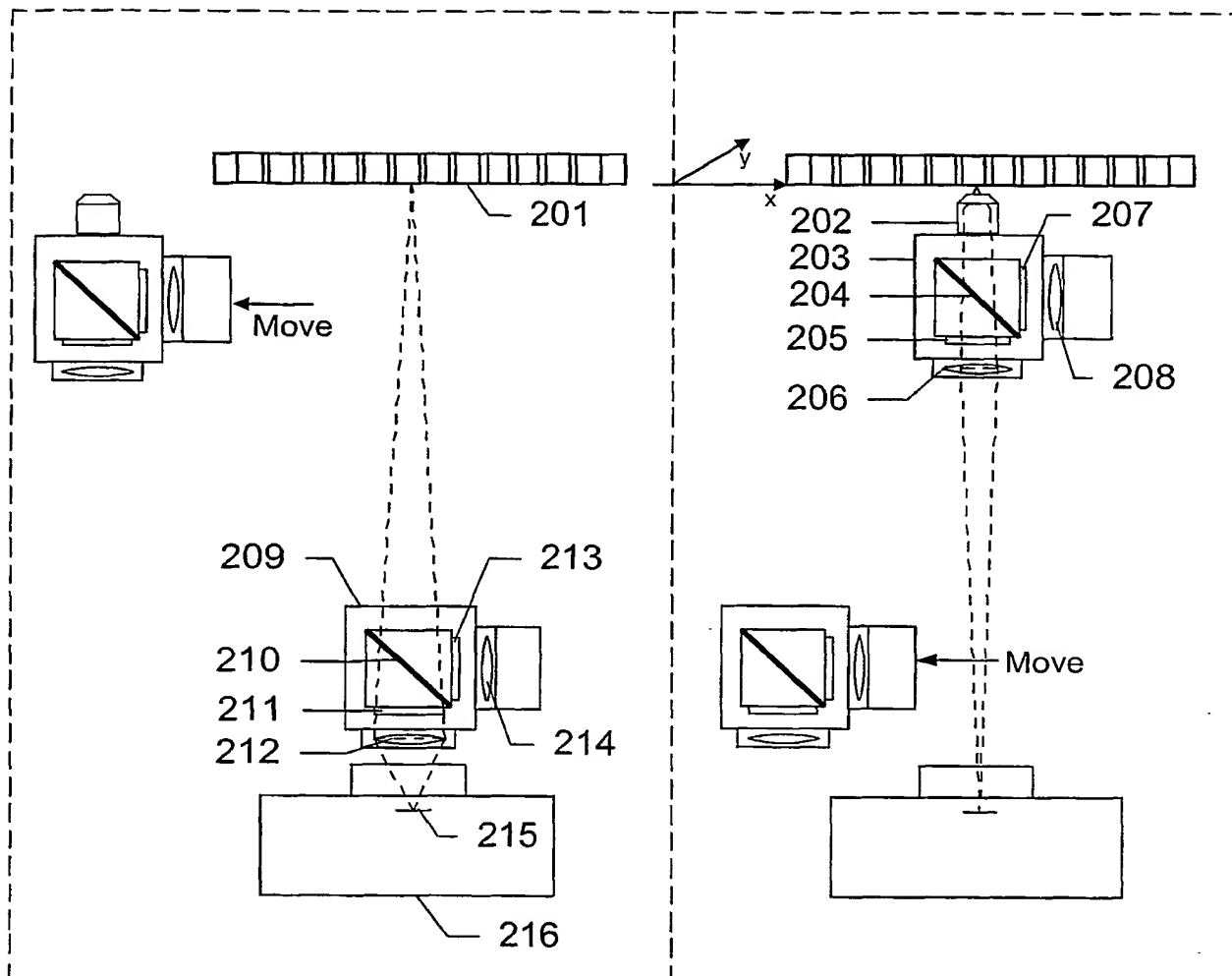


Figure 8

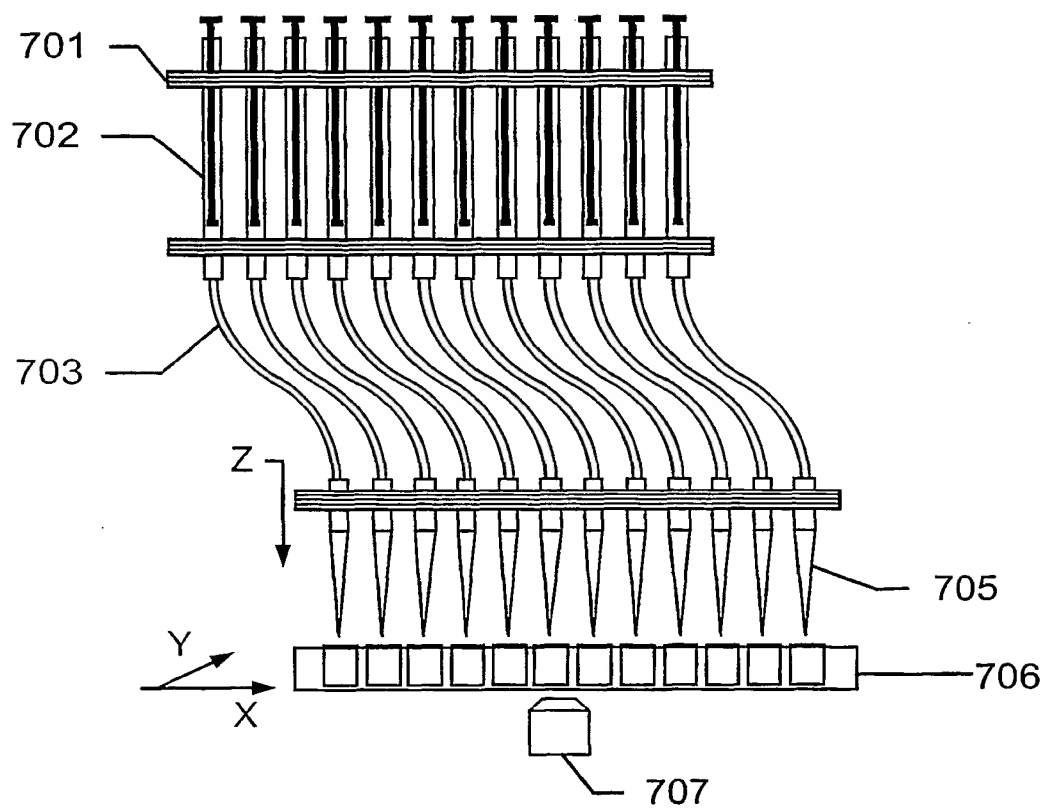


Figure 9

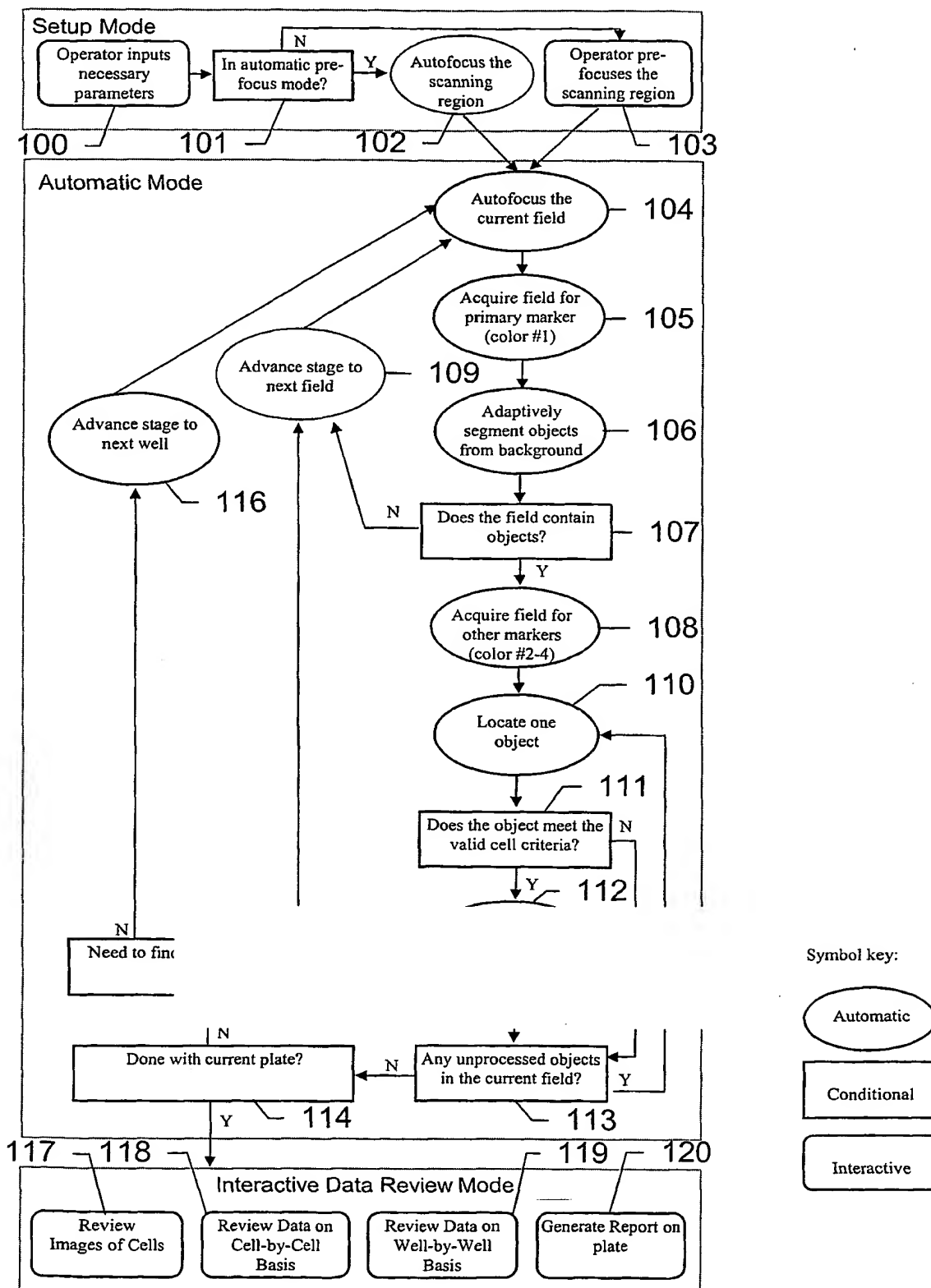


Figure 10

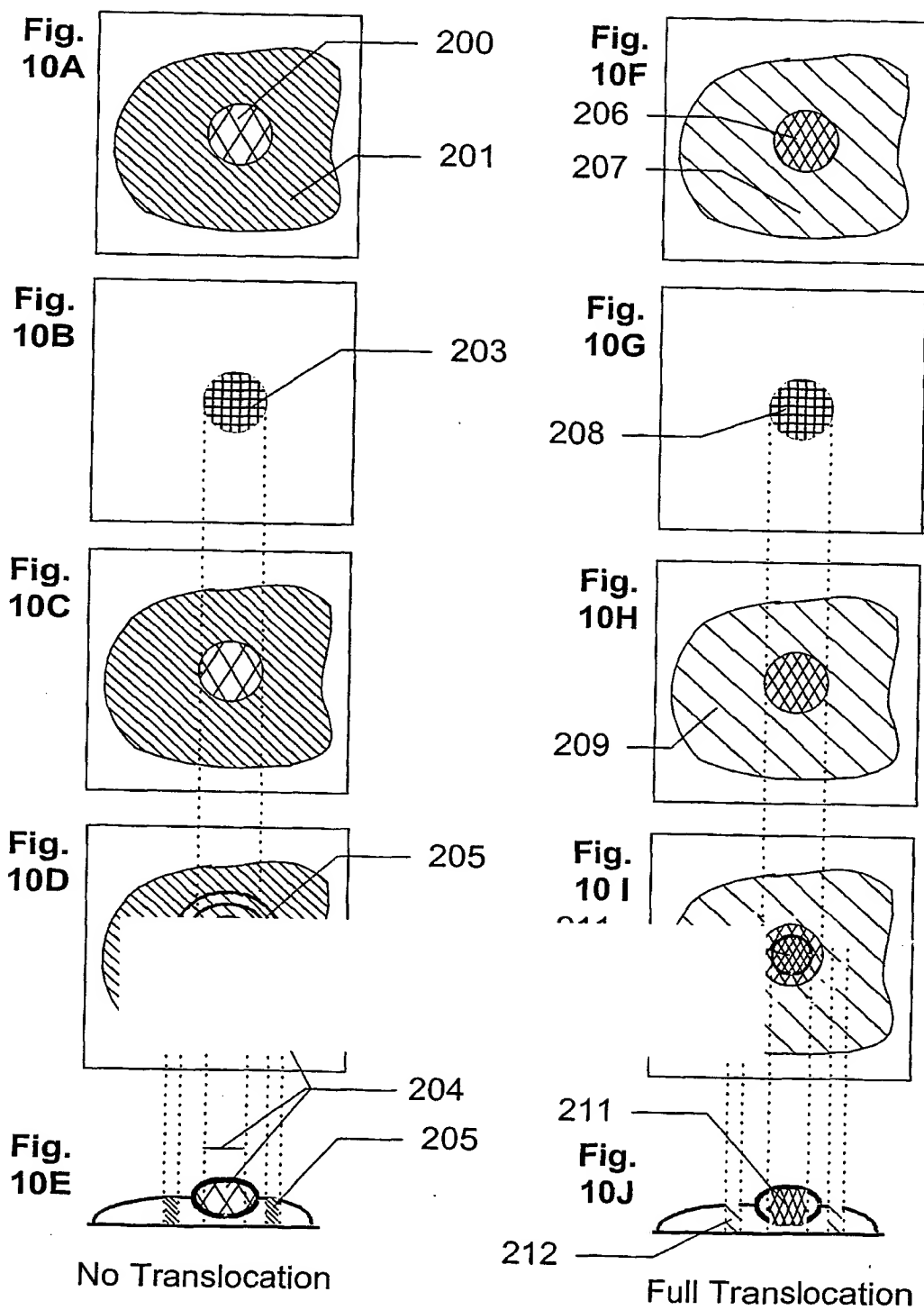


Figure 11

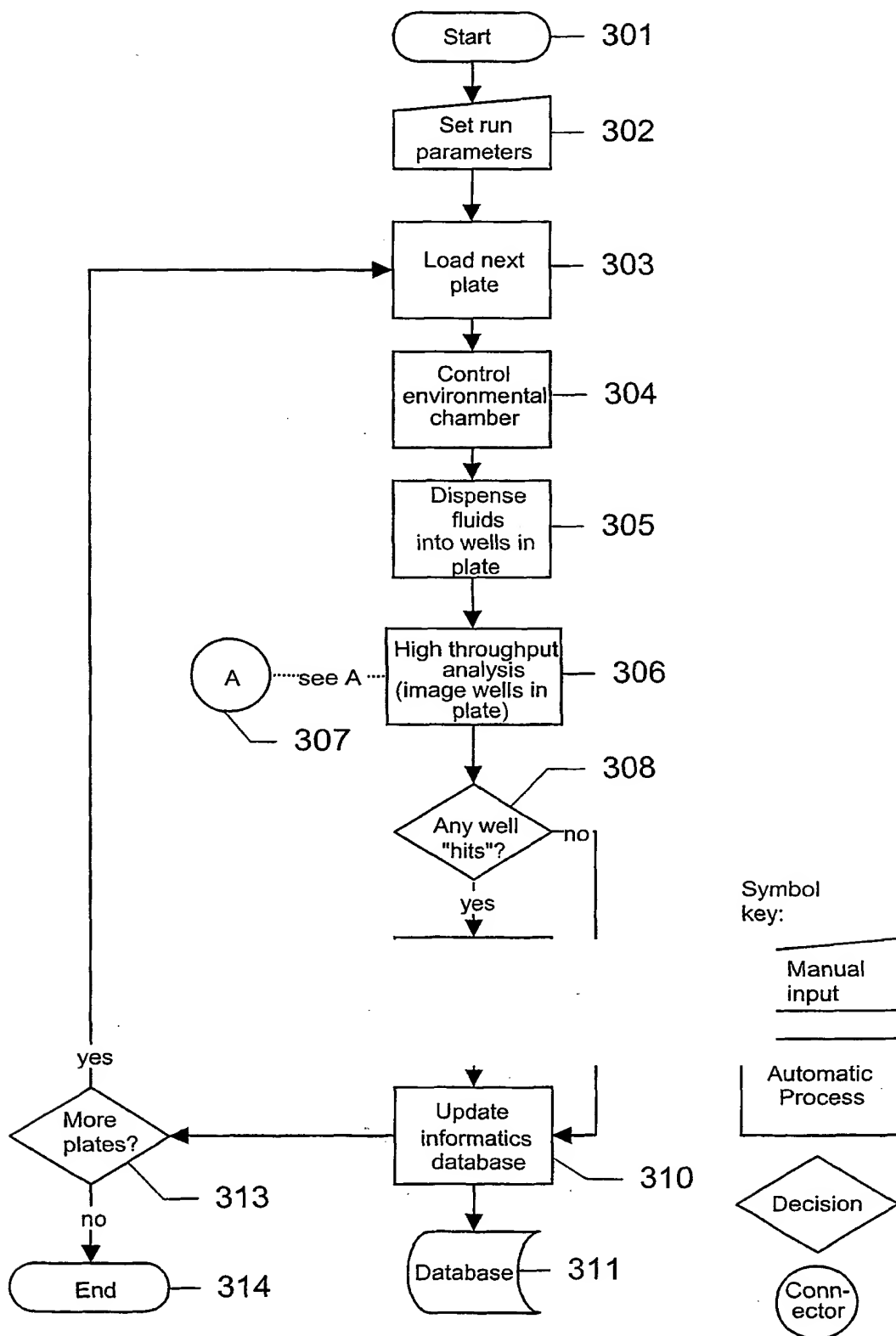


Figure 12

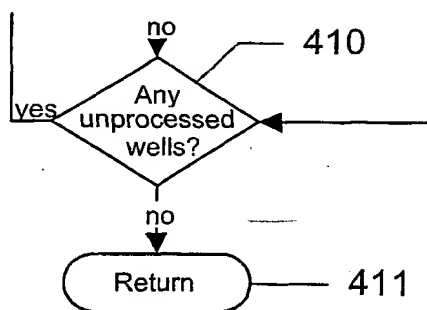
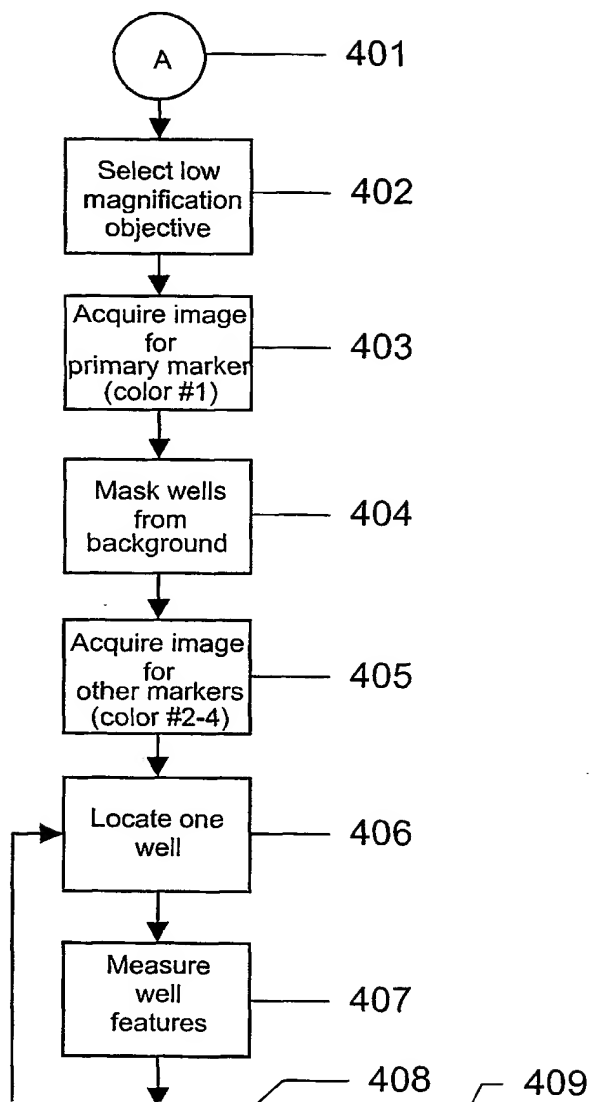


Figure 13

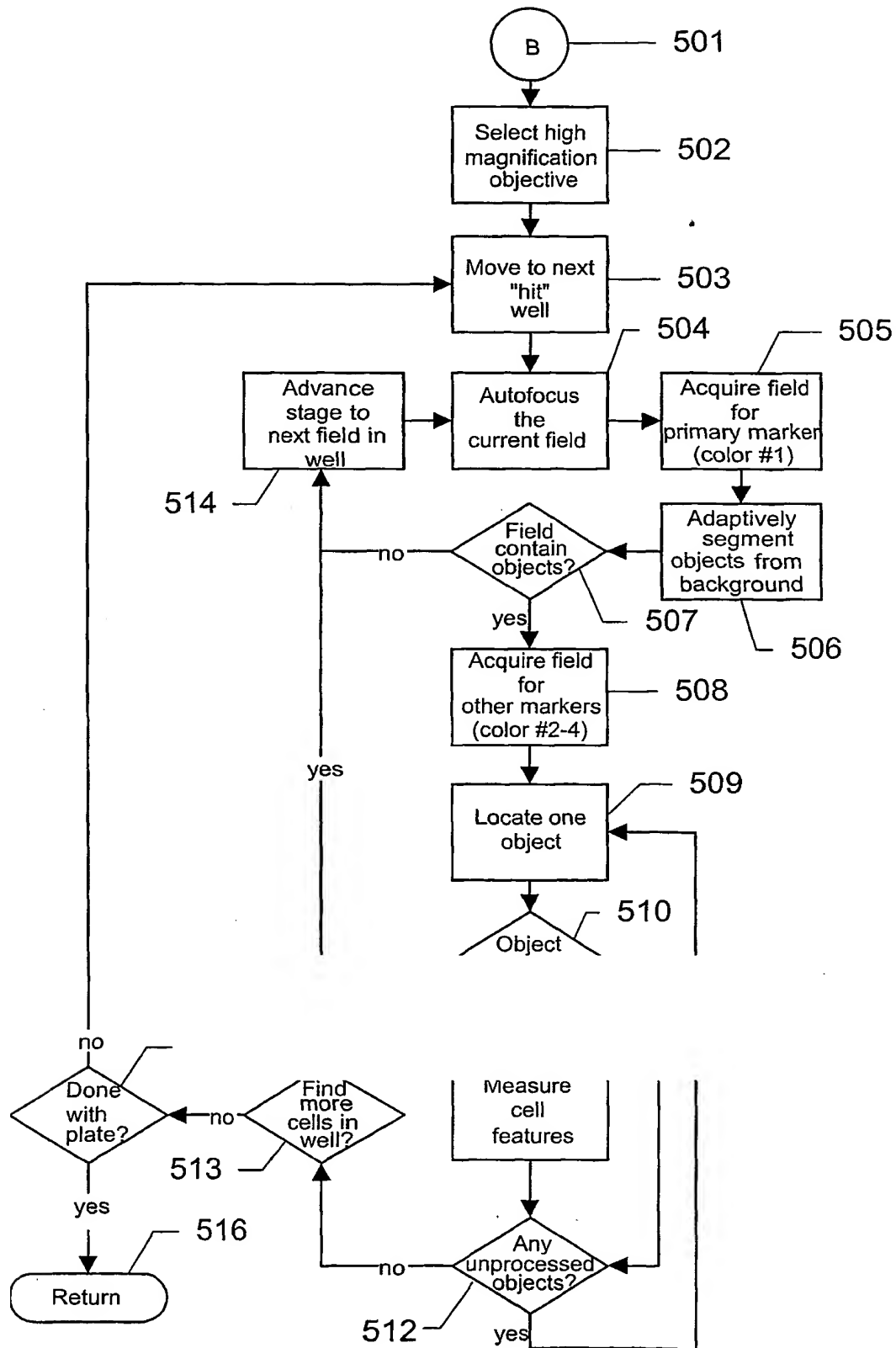


Figure 14

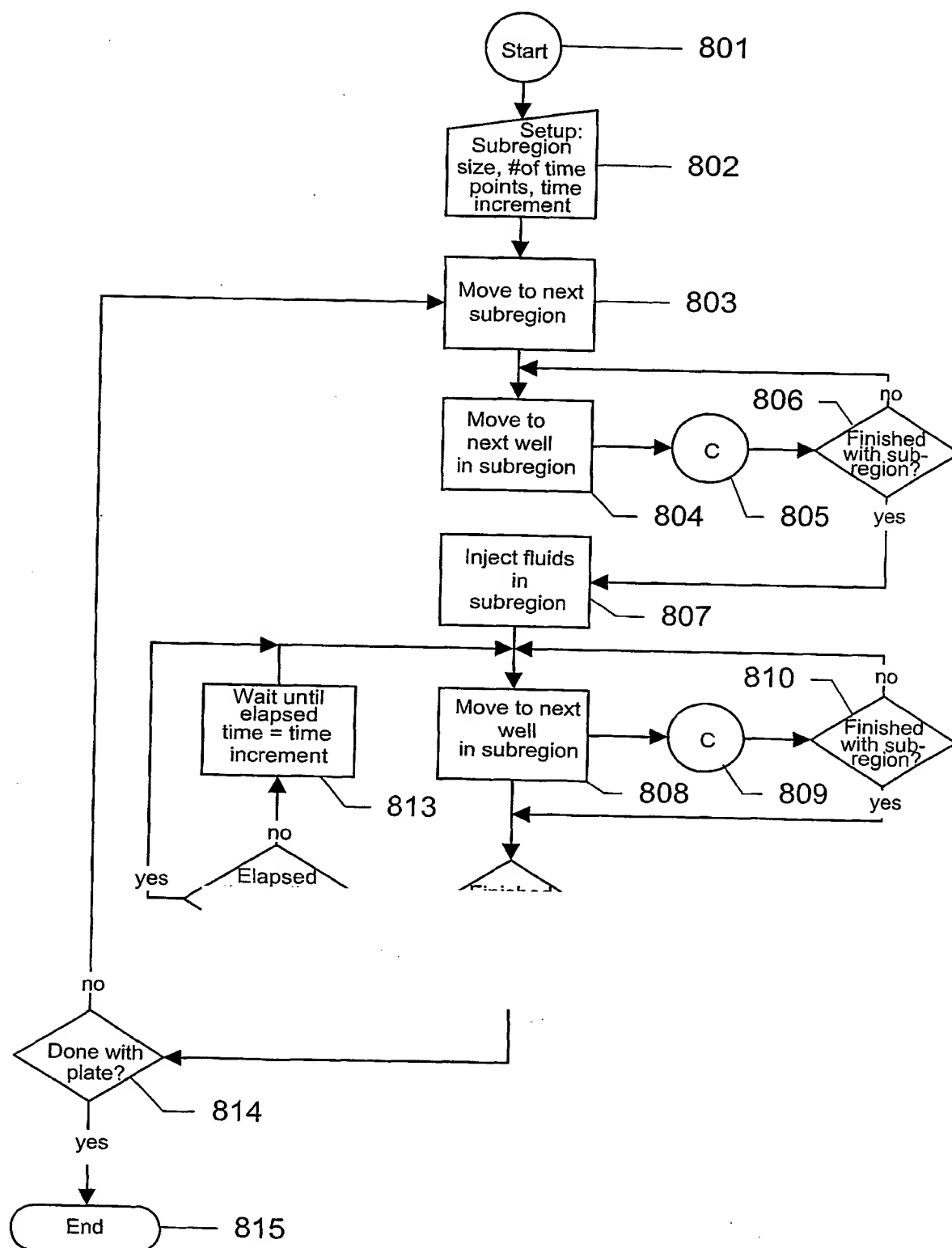


Figure 15

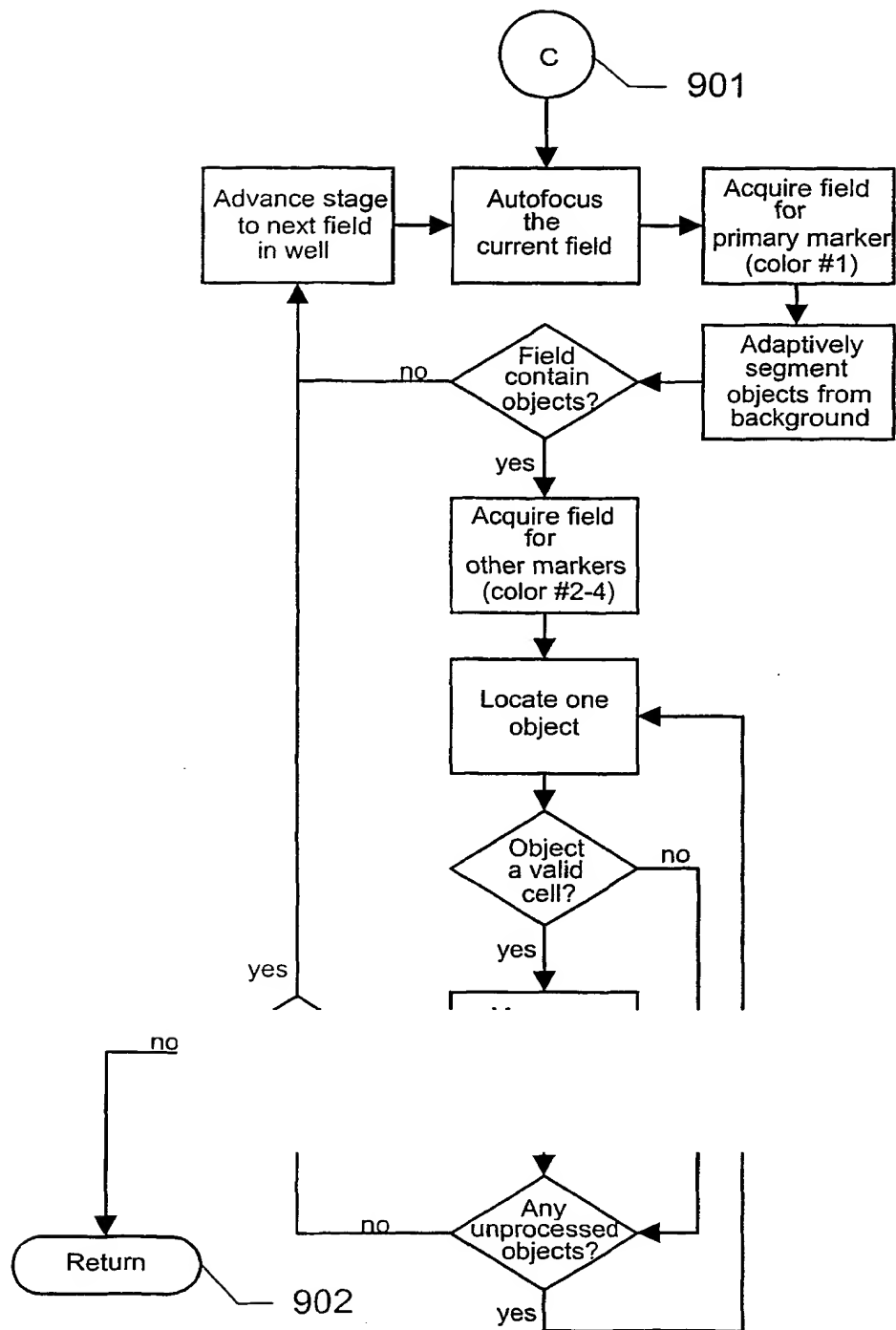


Figure 16

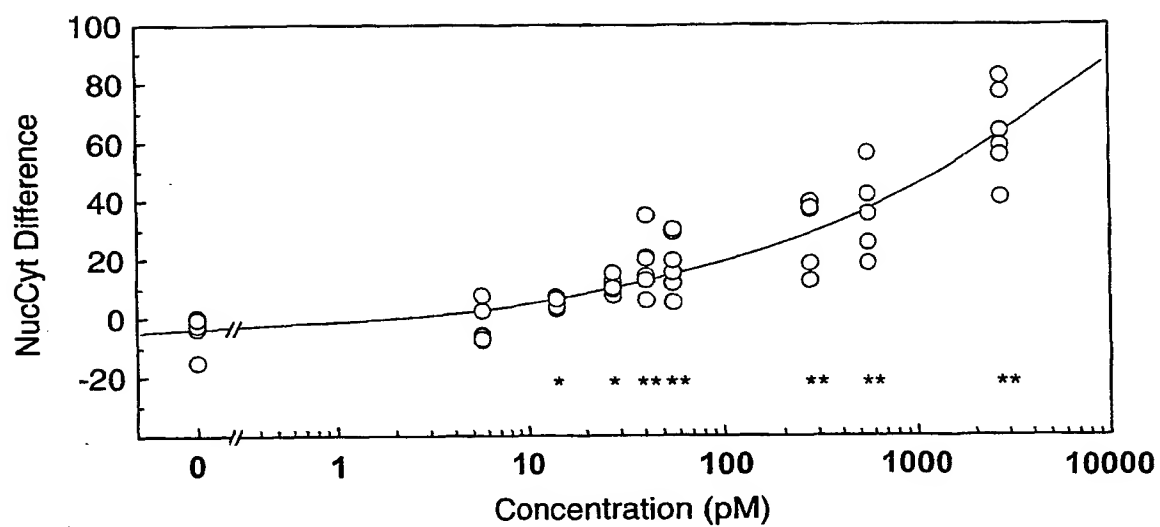


Figure 17

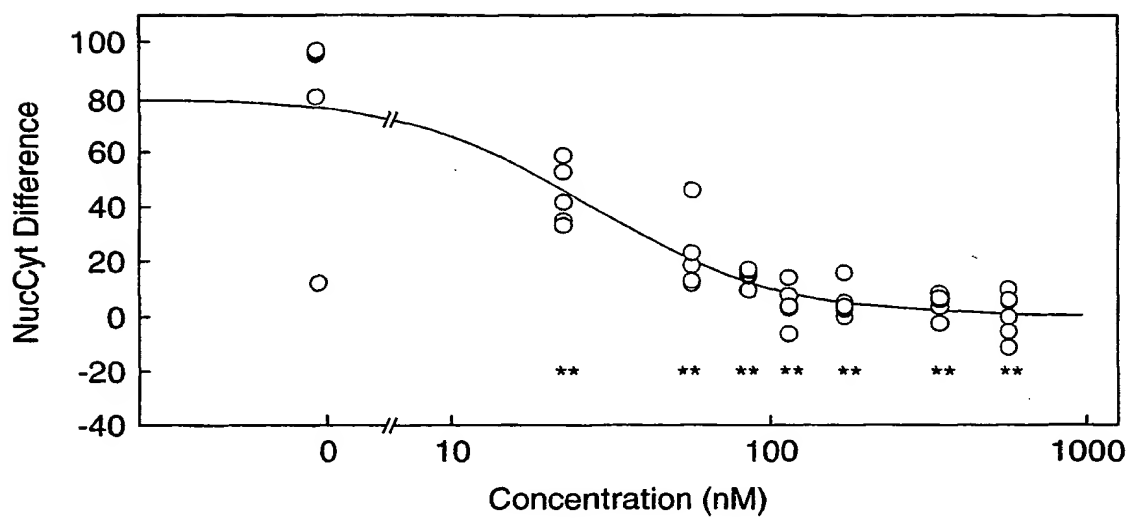


Figure 18

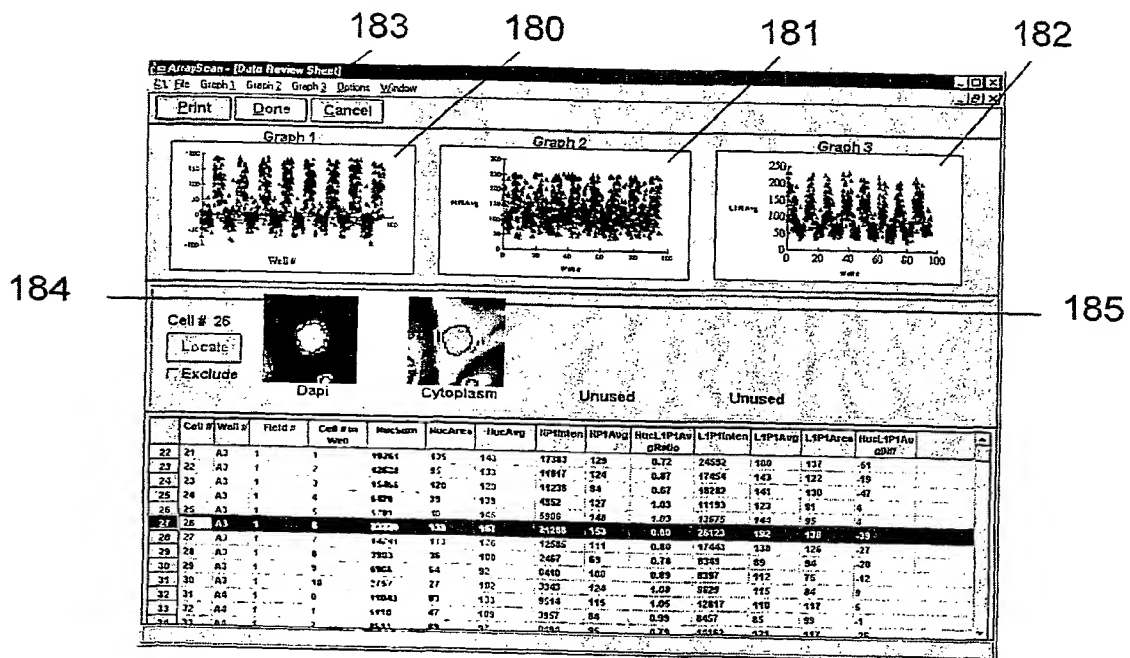


Figure 19

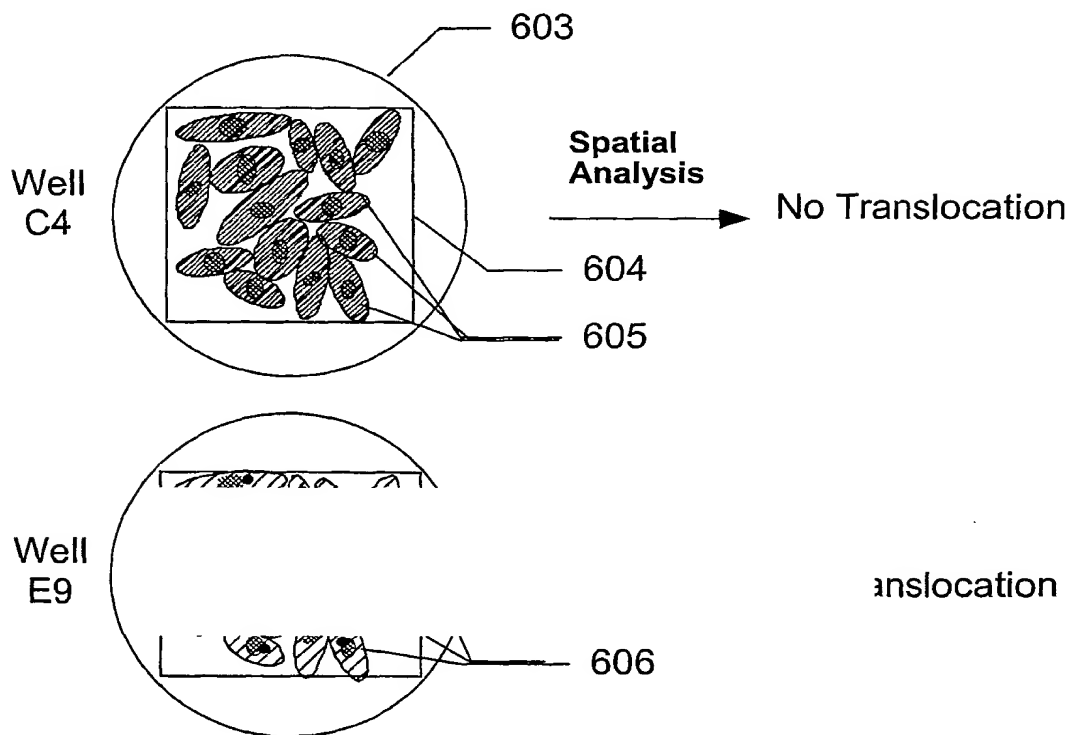
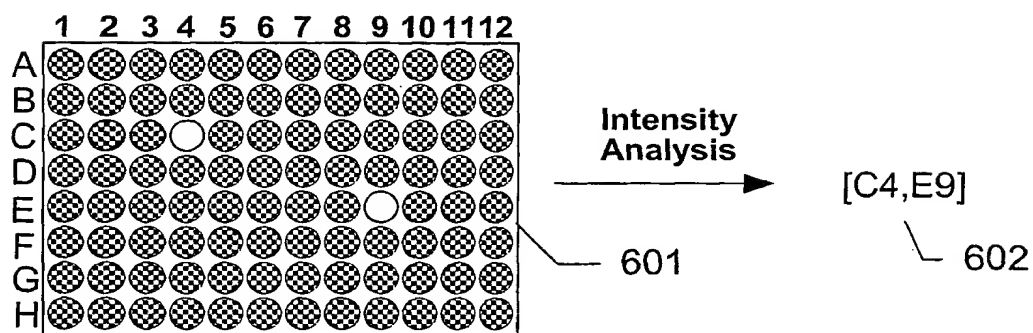


Figure 20

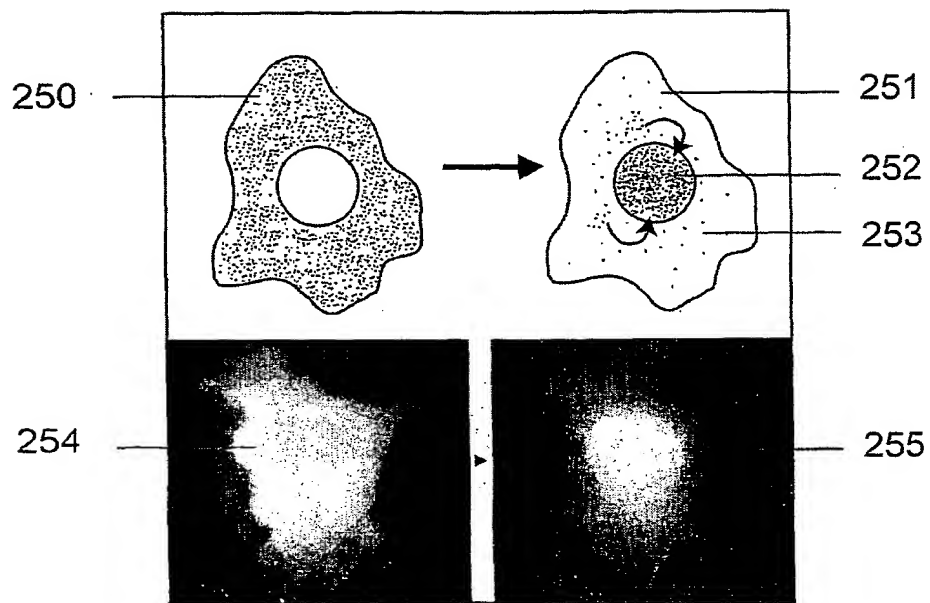


Fig. 20A

Fig. 20B

Figure 21

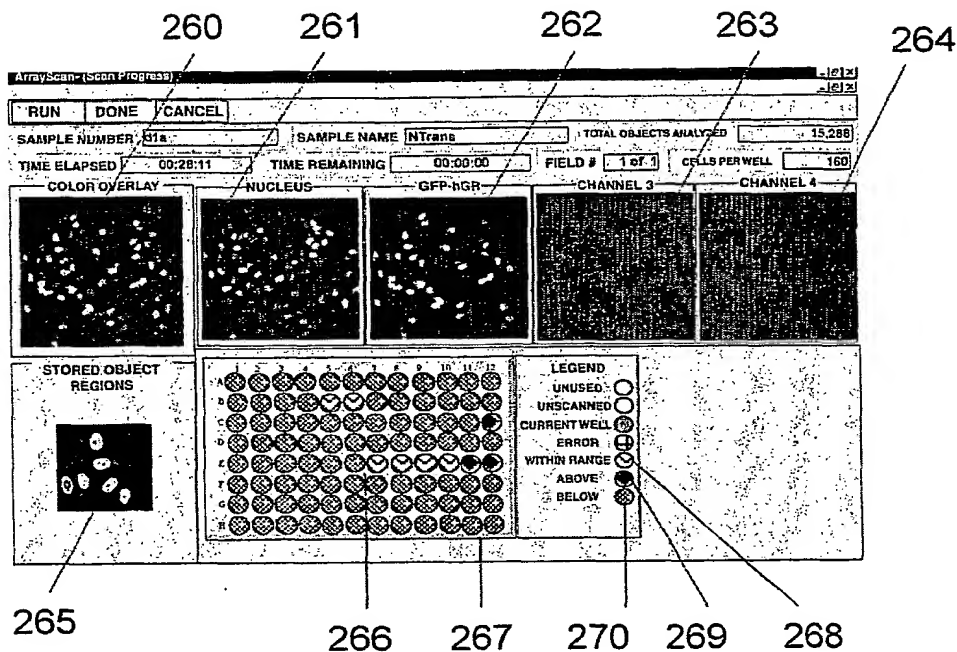


Figure 22

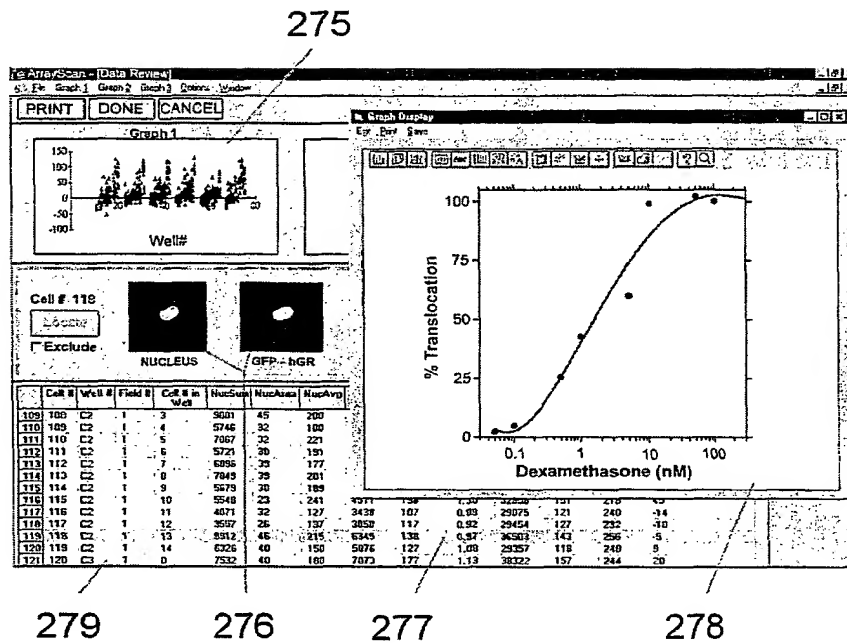


Figure 23

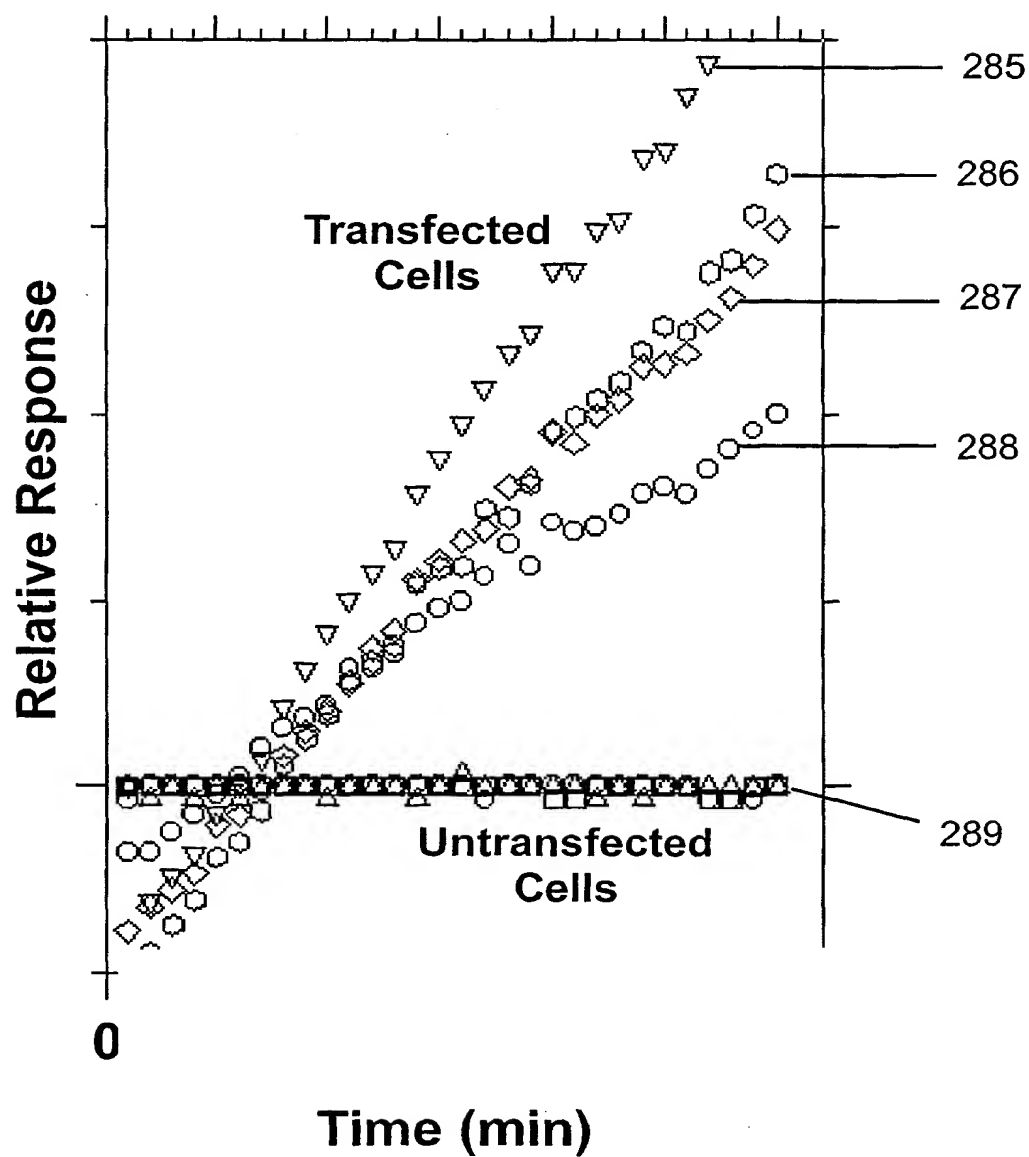


Figure 24

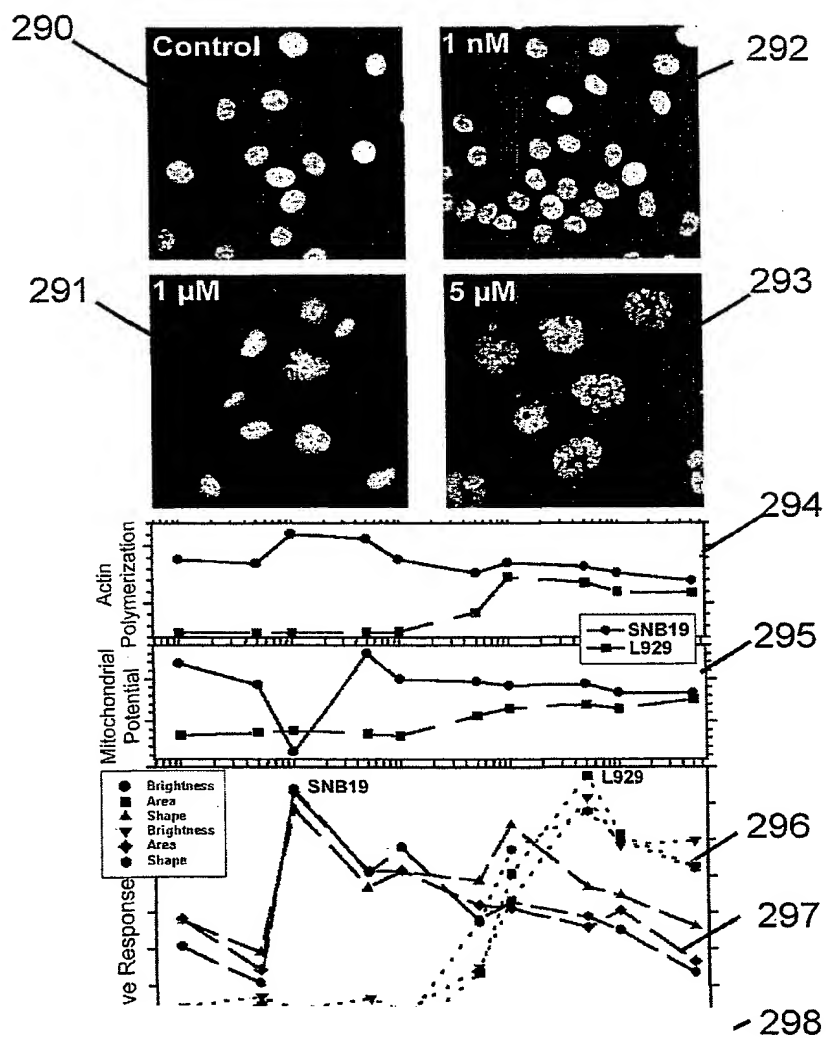


Figure 25

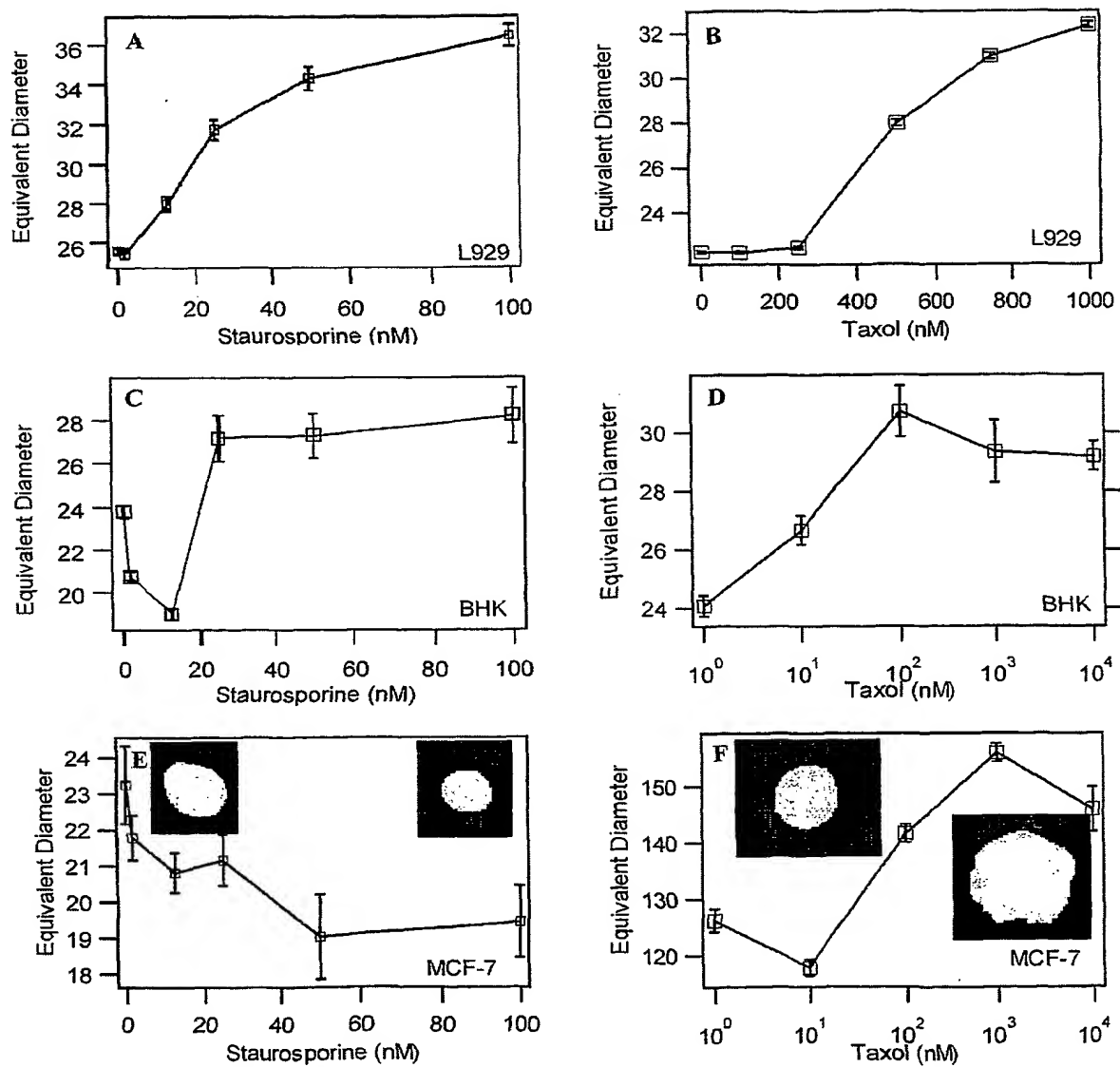


Figure 26

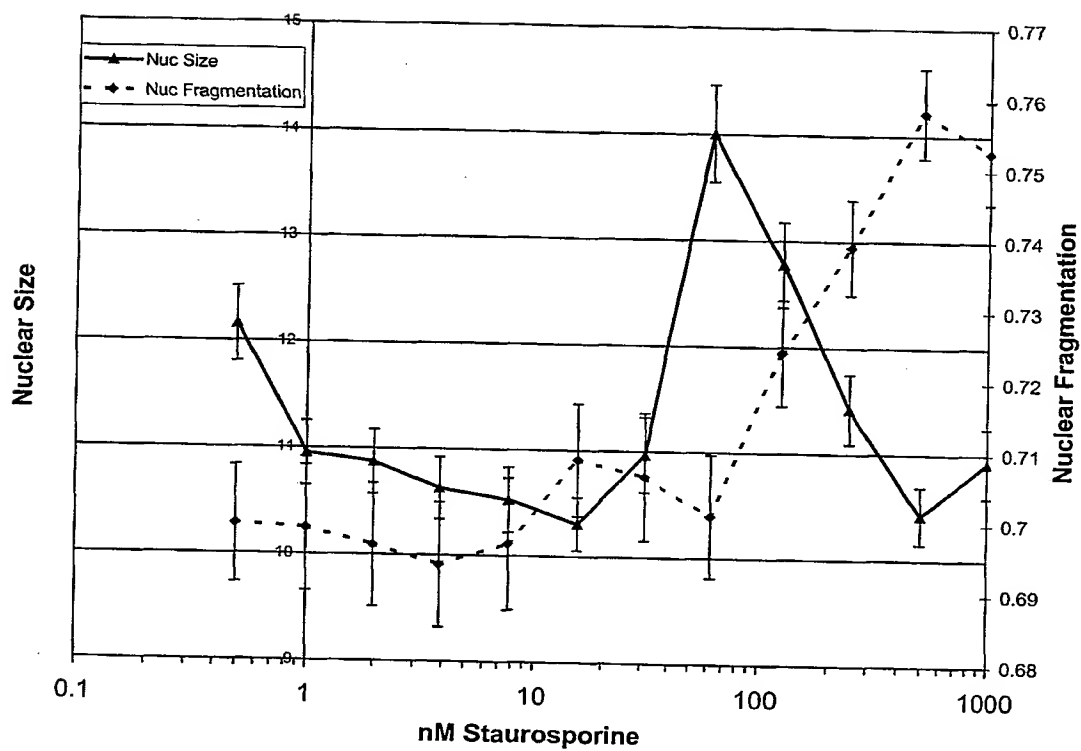


Figure 27

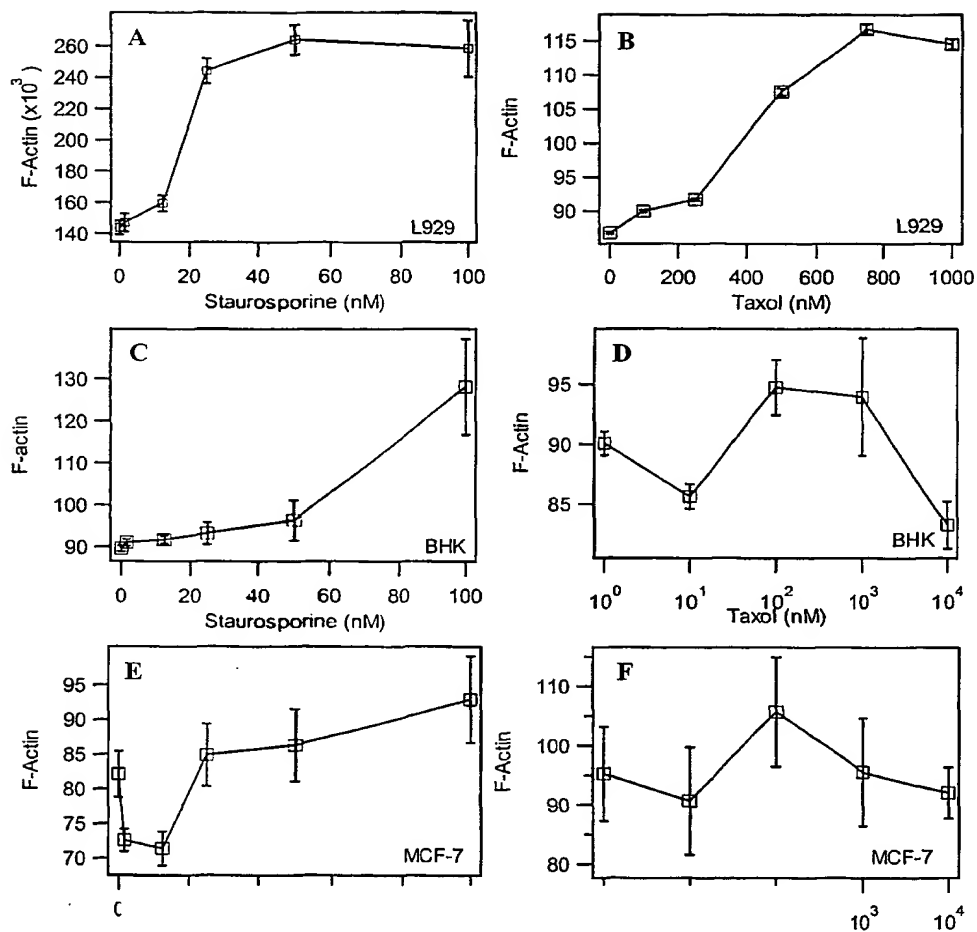


Figure 28

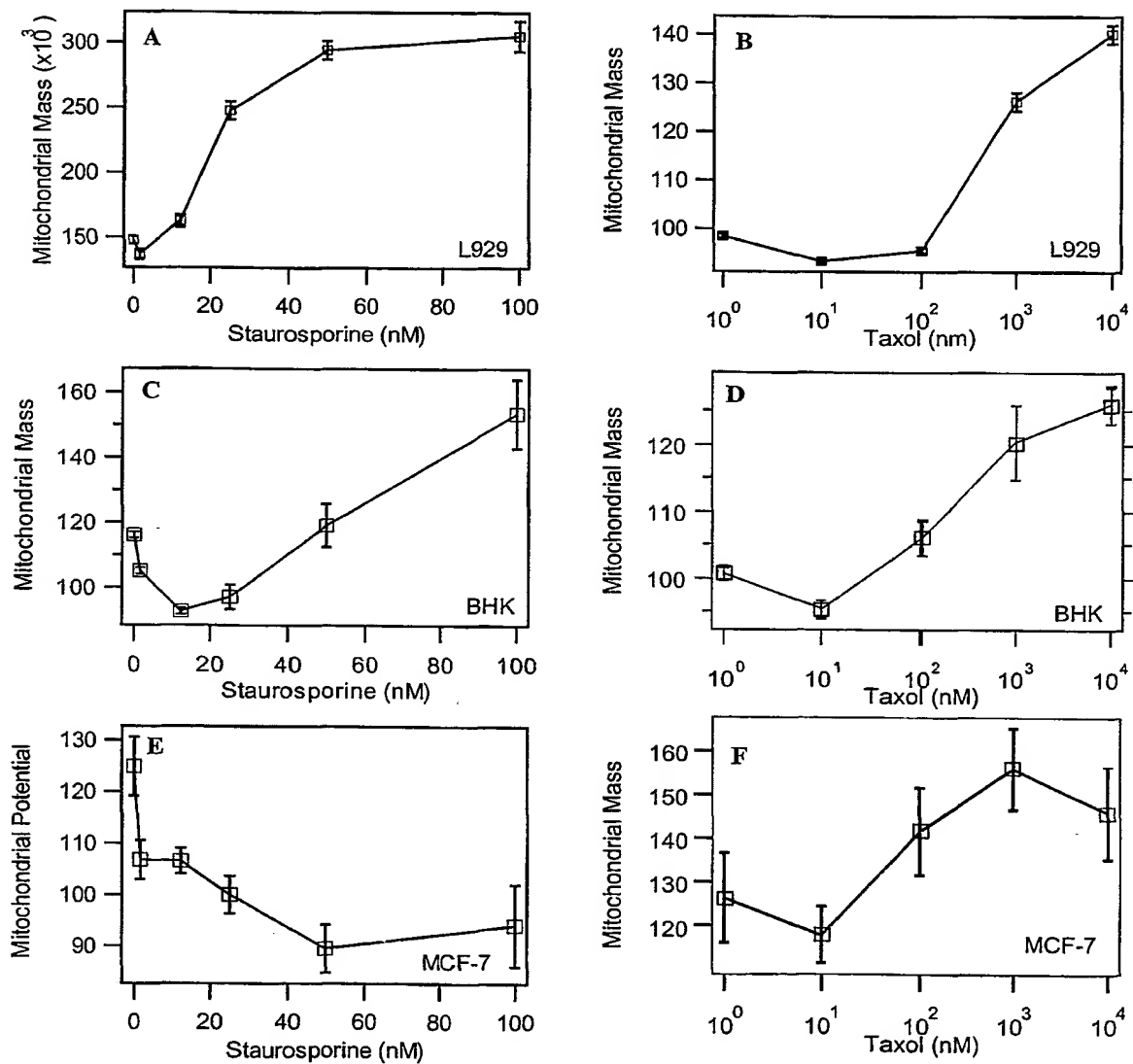


Figure 28G

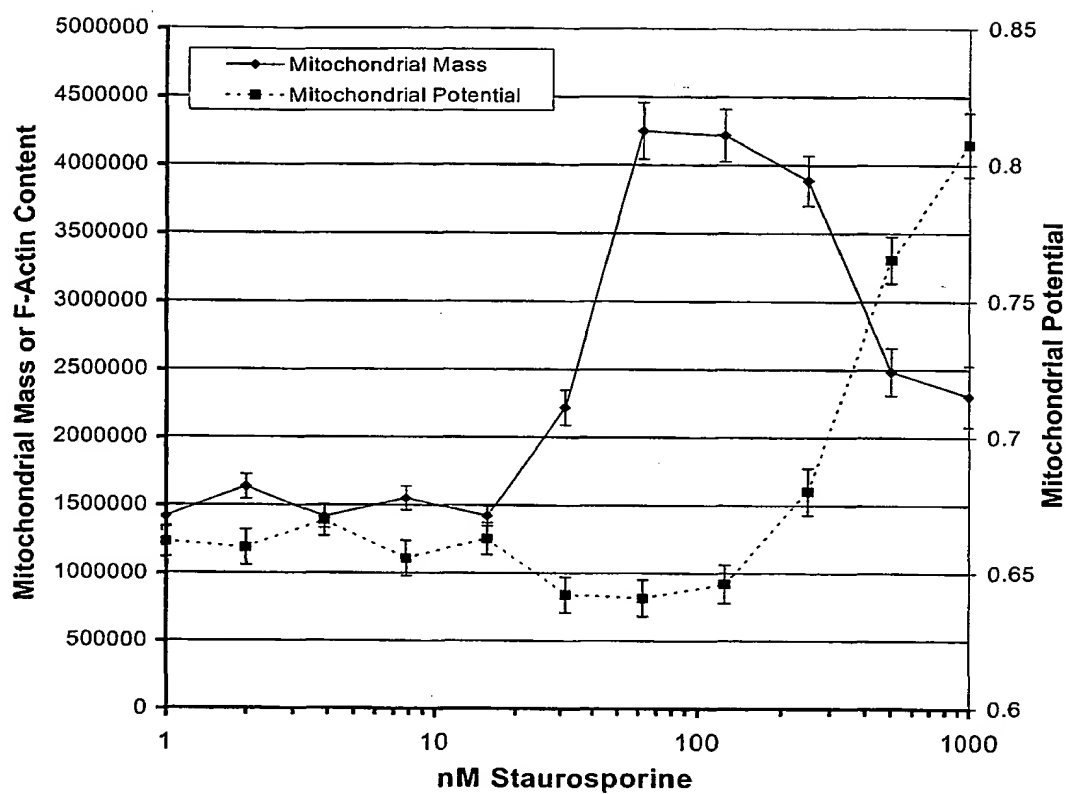


FIGURE 29A**1. SIGNAL SEQUENCES**

EPITOPE	SEQUENCE	SEQ ID NO:	REFERENCE
FLAG epitope	5' GACTACAAAGACGACG	35	Kasir, et al., 1999. J Biol Chem. 274:24873-80.
	AA Seq: ACGACAAA	36	
HA epitope	5' TACCCATACGACGTACCAGACTACGCA	37	Smith, et al., 1999. J Biol Chem. 274:19894-900.
	AA Seq: YPYDVDPYA	38	
KT3 epitope	5' CCACCAGAACCAGAAACA	39	MacArthur and Walter. 1984. J Virol. 52:483-91.
	AA seq: PPEPET	40	
Myc epitope	5' GCAGAAGAACAAAAATTAATAAGCGAAGA AGACTTA	41	Gosney, et al., 1990. Anticancer Res. 10:623-8.
	AA Seq: AEEQKLISEEDL	42	

EYFP: SEQ ID NO: 43 (Nucleic acid); SEQ ID NO:44 (Amino acid)

```

M V S K G E E L F T G V V P I L V E L D
ATGGTGAGCAAG GGCGAGGAGCTG TTCACCGGGGTG GTGCCCATCCTG GTCGAGCTGGAC

G D V N G H K F S V S G E G E G D A T Y
GGCGACGTAAAC GGCCACAAGTTC AGCGTGTCCGGC GAGGGCGAGGGC GATGCCACCTAC

G K L T L K F I C T T G K L P V P W P T
GGCAAGCTGACC CTGAAGTTCATC TGCACCACCGGC AAGCTGCCCGTG CCCTGGCCCCACC

L V T T F G Y G L Q C F A R Y P D H M K
CTCGTGACCACC TTCGGCTACGGC CTGCAGTGCTTC GCGCGTACCCC GACCACATGAAG

Q H D F F K S A M P E G Y V Q E R T I F
CAGCACGACTTC TTCAAGTCCGCC ATGCCCCGAAGGC TACGTCCAGGAG CGCACCATCTTC

F K D D G N Y K T R A E V K F E G D T L
TTCAAGGACGAC GGCAACTACAAG ACCCGCGCCGAG GTGAAGTTCGAG GGCGACACCCTG

V N R I E H
GTGAACCGCATC GA GGCAC

K L E Y N N
AAGCTGGAGTAC AACTACAACAGC CACAACGTCTAT ATCATGGCCGAC AAGCAGAAGAAC

G I K V N F K I R H N I E D G S V Q L A
GGCATCAAGGTG AACTTCAAGATC CGCCACAACATC GAGGACGGCAGC GTGCAGCTCGCC

D H Y Q Q N T P I G D G P V L L P D N H
GACCACTACCAG CAGAACACCCCC ATCGGCGACGGC CCCGTGCTGCTG CCCGACAACCAC

Y L S Y Q S A L S K D P N E K R D H M V
TACCTGAGCTAC CAGTCCGCCCTG AGCAAAGACCCC AACGAGAAGCGC GATCACATGGTC

```

L L E F V T A A G I T L G M D E L Y K
CTGCTGGAGTTC GTGACCGCCGCC GGGATCACTCTC GGCATGGACGAG CTGTACAAG

EGFP: SEQ ID NO:45 (Nucleic acid); SEQ ID NO:46 (Amino acid)

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GGCGACGTAAAC GGCCACAAGTTC AGCGTGTCGGC GAGGGCGAGGGC GATGCCACCTAC

G K L T L K F I C T T G K L P V P W P T
GGCAAGCTGACC CTGAAGTTCATC TGCACCACCGGC AAGCTGCCCCGTG CCCTGGCCCACC

L V T T L T Y G V Q C F S R Y P D H M K
CTCGTGACCACC CTGACCTACGGC GTGCAGTGCTTC AGCCGCTACCCC GACCACATGAAG

Q H D F F K S A M P E G Y V Q E R T I F
CAGCACGACTTC TTCAAGTCCGCC ATGCCCCGAAGGC TACGTCCAGGAG CGCACCATCTTC

F K D D G N Y K T R A E V K F E G D T L
TTCAAGGACGAC GGCAACTACAAG ACCCGCGCCGAG GTGAAGTTCGAG GGCGACACCCTG

V N R I E L K G I D F K E D G N I L G H
GTGAACCGCATC GAGCTGAAGGGC ATCGACTTCAAG GAGGACGGCAAC ATCCTGGGGCAC

K L E Y N Y N S H N V Y I M A D K Q K N
AAGCTGGAGTAC AACTACAACAGC CACAACGTCTAT ATCATGGCCGAC AAGCAGAAGAAC

G I K V N F K I R H N I E D G S V Q L A
GGCATCAAGGTG AACTTCAAGATC CGCCACAACATC GAGGACGGCAGC GTGCAGCTCGCC

D H Y Q Q N T P I G D G P V L L P D N H
GACCACTACCAG CAGAACACCCCC ATCGGCGACGGC CCCGTGCTGCTG CCCGACAACCAC

Y L S T Q S A L S K D P N E K R D H M V
TACCTGAGCACC CAGTCCGCCCTG AGCAAAGACCCC AACGAGAAGCGC GATCACATGGTC

L L E F V T A A G I T L G M D E L Y K
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EBFP: SEQ ID NO:47 (Nucleic acid); SEQ ID NO:48 (Amino acid)

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GGCGACGTAAAC GGCCACAAGTTC AGCGTGTCGGC GAGGGCGAGGGC GATGCCACCTAC

G K L T L K F I C T T G K L P V P W P T
GGCAAGCTGACC CTGAAGTTCATC TGCACCACCGGC AAGCTGCCCCGTG CCCTGGCCCACC

L V T T L T H G V Q C F S R Y P D H M K
 CTCGTGACCACC CTGACCCACGGC GTGCAGTGCTTC AGCCGCTACCCC GACCACATGAAG
 Q H D F F K S A M P E G Y V Q E R T I F
 CAGCAGCACTTC TTCAAGTCCGCC ATGCCCCGAAGGC TACGTCCAGGAG CGCACCATCTTC
 F K D D G N Y K T R A E V K F E G D T L
 TTCAAGGACGAC GGCAACTACAAG ACCCGCGCCGAG GTGAAGTTCGAG GGCGACACCCTG
 V N R I E L K G I D F K E D G N I L G H
 GTGAACCGCATC GAGCTGAAGGGC ATCGACTTCAAG GAGGACGGCAAC ATCCTGGGGCAC
 K L E Y N F N S H N V Y I M A D K Q K N
 AAGCTGGAGTAC AACTTCAACAGC CACAACGTCTAT ATCATGGCCGAC AAGCAGAAGAAC
 G I K V N F K I R H N I E D G S V Q L A
 GGCATCAAGGTG AACTTCAAGATC CGCCACAACATC GAGGACGGCAGC GTGCAGCTCGCC
 D H Y Q Q N T P I G D G P V L L P D N H
 GACCACTACCAG CAGAACACCCCC ATCGGCGACGGC CCCGTGCTGCTG CCCGACAACCAC
 Y L S T Q S A L S K D P N E K R D H M V
 TACCTGAGCACC CAGTCCGCCCTG AGCAAAGACCCC AACGAGAAGCGC GATCACATGGTC
 L L E F V T A A G I T L G M D E L Y K
 CTGCTGGAGTTC GTGACCGCCGCC GGGATCACTCTC GGCATGGACGAG CTGTACAAG

ECFP: SEQ ID NO:49 (Nucleic acid); SEQ ID NO:50 (Amino acid)

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 G D V N G H K F S V S G E G E G D A T Y
 GGCGACGTAAAC GGCCACAAGTTC AGCGTGTCCGGC GAGGGCGAGGGC GATGCCACCTAC
 G K L T L K F I C T T G K L P V P W P T
 GGCAAGCTGACC CTGAAGTTCATC TGCACCACCGGC AAGCTGCCCCGTG CCCTGGCCCCACC
 L V T T L T W G V Q C F S R Y P D H M K
 CTCGTGACCACC CTGACCTGGGGC GTGCAGTGCTTC AGCCGCTACCCC GACCACATGAAG
 Q H D F F K S A M P E G Y V Q E R T I F
 CAGCAGCACTTC TTCAAGTCCGCC ATGCCCCGAAGGC TACGTCCAGGAG CGCACCATCTTC
 F K D D G N Y K T R A E V K F E G D T L
 TTCAAGGACGAC GGCAACTACAAG ACCCGCGCCGAG GTGAAGTTCGAG GGCGACACCCTG
 V N R I E L K G I D F K E D G N I L G H
 GTGAACCGCATC GAGCTGAAGGGC ATCGACTTCAAG GAGGACGGCAAC ATCCTGGGGCAC
 K L E Y N Y I S H N V Y I T A D K Q K N
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 G I K A N F K I R H N I E D G S V Q L A

GGCATCAAGGCC AACTTCAAGATC CGCCACAACATC GAGGACGGCAGC GTGCAGCTCGCC
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 Y L S T Q S A L S K D P N E K R D H M V
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 L L E F V T A A G I T L G M D E L Y K
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Fred25: SEQ ID NO:51 (Nucleic acid); SEQ ID NO:52 (Amino acid)

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 G K L T L K F I C T T G K L P V P W P T
 GGAAAACCTTACC CTGAAGTTCATC TGCACTACTGGC AAAGTGCCTGTT CCATGGCCAACA
 L V T T L C Y G V Q C F S R Y P D H M K
 CTAGTCACTACT CTGTGCTATGGT GTTCAATGCTTT TCAAGATACCCG GATCATATGAAA
 R H D F F K S A M P E G Y V Q E R T I F
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 F K D D G N Y K T R A E V K F E G D T L
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 V N R I E L K G I D F K E D G N I L G H
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 G I K V N F K T R H N I E D G S V Q L A
 GGAATCAAAGTG AACTTCAAGACC CGCCACAACATT GAAGATGGAAGC GTTCAACTAGCA
 D H Y Q Q N T P I G D G P V L L P D N H
 GACCATTATCAA CAAAATACTCCA ATTGCGGATGGC CCTGTCCTTTTA CCAGACAACCAT
 Y L S T Q S A L S K D P N E K R D H M V
 TACCTGTCCACA CAATCTGCCCTT TCGAAAGATCCC AACGAAAAGAGA GACCACATGGTC
 L L E F V T A A G I T H G M D E L Y N *
 CTTCTTGAGTTT GTAACAGCTGCT GGGATTACACAT GGCATGGATGAA CTGTACAACATG

FIGURE 29B

2. PROTEASE RECOGNITION SITES

Substrate Recognitions Sequences	Source	Recognition Site	SEQ ID NO	Reference
Caspase-1,4,5	peptide library	5'(TGG,TTA)GAACATGACAA Seq:(W,L)EHD/	53 54	Thornberry et al., 1997, J. Biol. Chem. 272:17907
proCaspase-1	peptide library	5'TGGTTTAAAGAC AA Seq: WFKD/	55 56	Thornberry et al., 1997, J. Biol. Chem. 272:17907
Caspase-2	peptide library	5'GACGAACACGAC AA Seq: DEHD/	57 58	Thornberry et al., 1997, J. Biol. Chem. 272:17907
Caspase 3, 7	PARP	5'GACGAAGTTGAC AA Seq: DEVD/	59 60	Benekke, et al., 1997. Biochem Mol Biol Int. 43:755-61; Thornberry et al., 1997, J. Biol. Chem. 272:17907
ProCaspase 3	Caspase-3	5'ATAGAAACAGAC AA Seq: IETD/	61 62	Tewari, M., et al., 1995. Cell. 81:801-9.
ProCaspase-4,5	peptide library	5'TGGGTAAGAGAC AA Seq: WVRD/	63 64	Thornberry, N.A. et al., 1997, J.Biol. Chem. 272, 17907-17911
Caspase 6	Lamin A, peptide library	5'GTAGAAATAGAC AA Seq: VEID/ 5'GTAGAACACGAC AA Seq: VEHD/	65 66 67 68	Nakajima and Sado. 1993. Biochim Biophys Acta. 1171:311-4; Thornberry et al., 1997, J. Biol. Chem. 272:17907
proCaspase 6	Caspase-6	5'ACAGAAAGTAGAC AA Seq: TEVD/	69 70	Fernandes-Alnemri, et al., 1994. J Biol Chem. 269:30761-4.
proCaspase-7	peptide library	5'ATACAAGCAGAC AA Seq: IQAD/	71 72	Thornberry, N.A. et al., 1997, J.Biol. Chem. 272, 17907-17911
Caspase 8	peptide library	5'GTAGAAACAGAC AA Seq: VETD/	73 74	Muzio, M., et al., 1996. Cell. 85:817-27; Fernandes-Alnemri, et al., 1996. Proc Natl Acad Sci U S A. 93:7464-9;Thornberry et al., 1997, J. Biol. Chem. 272:17907
proCaspase-8	Caspase-8	5'TTAGAAACAGAC AA Seq: LETD/	75 76	Muzio, M., et al., 1996. Cell. 85:817-27; Fernandes-Alnemri, et al., 1996. Proc Natl Acad Sci U S A. 93:7464-9;Thornberry et al., 1997, J. Biol. Chem. 272:17907
Caspase 9	peptide library	5'TTAGAACACGAC AA Seq: LEHD/	77 78	Thornberry, N.A. et al., 1997, J.Biol. Chem. 272, 17907-17911
proCaspase 9	Caspase-9	5'TTAGAACACGAC AA Seq: LEHD/	79 80	Thornberry, N.A. et al., 1997, J.Biol. Chem. 272, 17907-17911
HIV protease		5'AGCCAAAATTAC AA Seq: SQNY/ 5'CCAATAGTACAA AA Seq: PIVQ/	81 82 83 84	Matayoshi, et al., 1990. Science. 247:954-8.
Adenovirus endopeptidase		5'AUGTTTGGAGGA AA Seq: MFGG/ 5'GCAAAAAAAGA AA Seq: AKKR/	85 86 87 88	Weber and Tihanyi. 1994. Methods Enzymol. 244:595-604.
b-Secretase	Amyloid precursor protein	5'GTAAAAAUG AA Seq: VKM/ 5'GACGCAGAATTC DAEF/	89 90 91 92	Hardy et al., 1994, in Amyloid Protein Precursor in Development, Aging, and Alzheimer's Disease, ed. C.L. Masters et al., pp. 190-198.
Cathepsin D		5'AAACCAGCATTATTC AA Seq: KPALF 5'TTCAGATTA AA Seq: FRL/	93 94 95 96	Dunn, et al., 1998. Adv Exp Med Biol. 436:133-8.
Matrix Metalloproteases		5'GGACCATTAGGACCA AA Seq: GPLGP	97 98	Bouvier et al., 1993; Garbett et al., 1999; Hill and Sakanari, 1997; Kojima et al., 1998; Tyagi et al., 1995; Wilhelm et al., 1993; Williams and Auld, 1986; Haugland, R., Handbook of fluorescent probes and research Chemicals 7th ed.
Granzyme B	peptide library	5'ATAGAACAGAC AA Seq: IEPD/	99 100	Thornberry et al., 1997, J. Biol. Chem. 272:17907

Anthrax protease	MEK1	5'ATGCCCAAGAAGAAGCCGAC GCCCATCCAGCTGAACCC	101	Vitale et al., (1998) Biochem Biophys Res Commun 248 (3), 706-711
		AA Seq: MPKKKPTPIQLN	102	
Anthrax protease	MEK2	5'ATGCTGGCCCGGAGGAAGCCG GTGCTGCCGGCGCTCACCATCA ACCC	103	Vitale et al., (1998) Biochem Biophys Res Commun 248 (3), 706-711
		AA Seq: MLARRKPVLALTIN	104	
tetanus/botulinum	cellubrevin	5'GCCTCGCAGTTTGAAACA	105	McMahon et al., Nature 364:346- 349; Martin et al., J. Cell Biol. In press
		AA Seq: ASQFET	106	
tetanus/botulinum	synaptobrevin/ VAMP3	5'GCTTCTCAATTGAAACG	107	Schiavo et al., (1992) Nature 359, 832-5
		AA Seq: ASQFET	108	
Botulinum neurotoxin A	SNAP-25	5'GCCAACCAACGTGCAACA AA Seq: ANQ/RAT	109 110	Zhao, et al. Gene 145 (2), 313- 314 (1994)
Botulinum neurotoxin B	VAMP	5'GCTTCTCAATTGAAACG AA Seq: ASQ/FET	111 112	
Botulinum neurotoxin C	Syntaxin	5'ACGAAAAAAGCTGTGAAA AA Seq: TKK/AVK	113 114	Martin et al., J. Leukoc. Biol. 65 (3), 397-406 (1999)
Botulinum neurotoxin D	VAMP	5'GACCAGAAGCTCTGTGAG AA Seq: DQK/LSE	115 116	
Botulinum neurotoxin E	SNAP-25	5'ATCGACAGGATCATGGAG AA Seq: IDR/IME	117 118	
Botulinum neurotoxin F	VAMP	5'AGAGACCAGAAGCTCTCT AA Seq: RDQ/KLS	119 120	
Botulinum neurotoxin G	VAMP	5'ACGAGCGCAGCCAAGTTG AA Seq: TSA/AKL	121 122	

FIGURE 29C

3. PRODUCT/REACTANT TARGET SEQUENCES

Target	Target Source	Target domain (Product or Reactant)	SEQ ID NO	Reference
Cytoplasm/cytoskeleton	Annexin II	5'ATGTCTACTGTCCACGAAATCCTGTGCAAGCTCAGCTTGGAGGGTGTTCATTCTACACCCCCAAGTGCC3'	123	Eberhard, et al., 1997, Mol. Biol. Cell 8:293a.
		(Amino acid seq: M S T V H E I L C K L S L E G V H S T P P S A)	124	
Inner surface of plasma membrane	farnesylation	5'AUGGGATCTACATTAAGCGCAGAAGACAAAGCAGCAGTAGAAAGAAGCAAAUAGATAGACAGAACTTATTAAGAGAAGACGGAGAAAAAGCTGCTAGA3'	125	Ferruccio G, et al., J. Biol. Chem. 274, 5843-5850, 1999
		(AA seq: M G C T L S A E D K A A V E R S K M I D R N L R E D G E K A A R	126	
Nucleus	NFkB p50	5'AGAAGGAAACGACAAAAAG	127	Henkel, T et al., Cell 68, 1121-1133, 1992
		(AA seq: R R K R Q K)	128	
Nucleolus	NOLP	5'AGAAAACGTATACGTACTTACCTCAAGTCCGTCAGGCGGATGAAAAGAAGTGGITTTGAGATGTCTCGACCTATTCTTCCACCTTACT	129	Ueki, et al., 1998. Biochem Biophys Res Commun. 252:97-102.
		(AA seq: R K R I R T Y L K S C R R M K R S G F E M S R P I P S H L T)	130	
Mitochondria	cytochrome c oxidase	5'ATGTCGGCTCCTGACGCCGCTGCTGCTGCGGGCTTGACAGGCTCGGCCGCGCTCCAGTGCCGCGCGCAAGATCCATTCTGTTG	131	Rizzuto, et al., 1989. J Biol Chem. 264:10595-600.
		(AA Seq: M S V L T P L L L R G L T G S A R R L P V P R A L I H S L)	132	
Nuclear Envelope	ODV-E66 & ODV-E25	5'AUGAGCATTGTTTTAATAATTGTTATTGGATTTTTTTAATATGTTTTTATATTAAAGCAACAGCAAAGATCCCAGAGTACCAGTTGAATTAAUG	133	Hong, T, et al. PNAS, 94, 4050-4055, 1997
		(AA Seq: M S I V L I I V I V I F L I C F L Y L S N S K D P R V P V E L M)	134	
Golgi	Calreticulin	5'ATGAGGCTTCGGGAGCCGCTCCTGAGCGGCAGCGCCGCGATGCCAGCGCGTCCCTACAGCGGGCCTGCCGCTGCTCGTGGCCGCTCTGCGCTCTGCACCTTGGCGTCAACCTCGTTTACTACCTGGCTGGCGCGACCTGAGCCGCGCTGCCCAACTGGTGGGAGTCTCCACACCGCTGCAGGGCGGCTCGAACAGTGCCGCCGCGCATCGGGCAGTCTCCGGGGAGCTCCGGACCGGAGGGGCC	135	Fliegel, L., et al., J. Biol. Chem. 264, 21522-21528, 1989.
		(AA Seq: M R L R E P L L S G S A A M P G A S L Q R A C R L L V A V C A L H L G V T L V Y Y L A G R D L S R L P Q L V G V S T P L Q G G S N S A A I G Q S S G E L R T G G A)	136	
Endoplasmic reticulum	D-AKAP1	5'GAAACAATAAGACCTATAAGAAGATGTAGTACATTTACATCTACAGACAGCAAAAUGGCAATTCAATTAAGATCTCCCTTCCATTAGCATTACCAGGAAUGTTAGCTTTATTAGGATGGTGGTGGTTTTTCAGTAGAAAAAAA	137	Huang, L.J. Et al., J. Cell. Biol. 145, 951-959, 1999
		(AA Seq: E T I R P I R I R C S Y F T S T D S K M A I Q L R S P F L A L P G M L A L L G W W W F F S R K K	138	
Nuclear Export	MEK1	5'GCCTTGCAAGAAGCTGGAGGAGCTAGAGCTTGATGAG	139	Fukuda, (1997) J. Biol. Chem 272, 51, 32642-32648
		(AA SEQ: A L Q K K L E E L E L D E	140	
Size exclusion	PROJ domain of MAP4	5'GCCGACCTCAGTCTTGTGGATGCGTTGACAGAACCACCTCCAGAAATTGAGGGAGAAATAA	141	West, (1991). J Biol Chem

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Vesicle membrane	Synaptobrevin	<p>5' ATGTGGGCAATCGGGATTACTGTTCT GGTTATCTTCATCATCATCATCATCGTG TGGGTTGTC</p> <p>(AA SEQ: M W A I G I T V L V I F I I I I I V W V V)</p>	143	Schiavo et al., (1992) Nature 359, 832-5
Vesicle membrane	Cellubrevin	<p>5' ATGTGGGCGATAGGGATCAGTGTCCCT GGTGATCATTTGTCATCATCATCATCGTG TGGTGTG</p> <p>(AA SEQ: M W A I G I S V L V I I V I I I I V W C)</p>	144 145 146	McMahon et al., Nature 364:346-349; Martin et al., J. Cell Biol. In press
Nuclear Export	MEK2	<p>5' GACCTGCAGAAGAAGCTGGAGGAGCT GGAACCTGACGAG</p> <p>AA SEQ: DLQKKLEBLELDE</p>	147 148	Zheng and Guan, J. Biol. Chem. 268:11435-11439, 1993

Peroxisome	PX	5'TCTAAACTG AA SEQ: S K L	149 150	Amery et al., Biochem. J. 336:367-371 (1998)

Microtubules (MAP4) SEQ ID NO:151 (Nucleic acid); SEQ ID NO:152 (amino acid)

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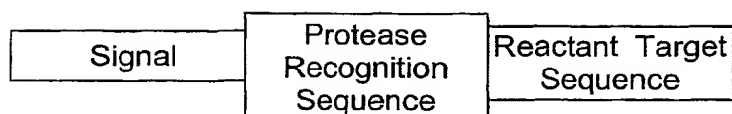
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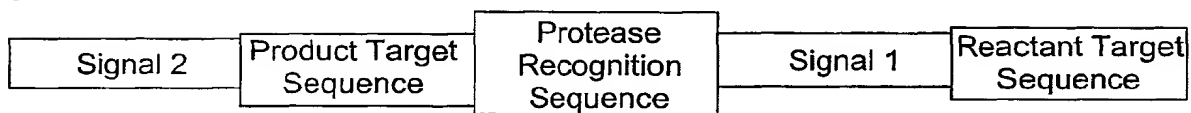
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Figure 30

A



B



C

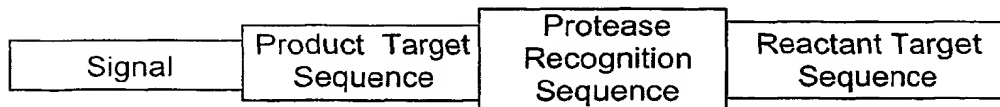


Figure 31

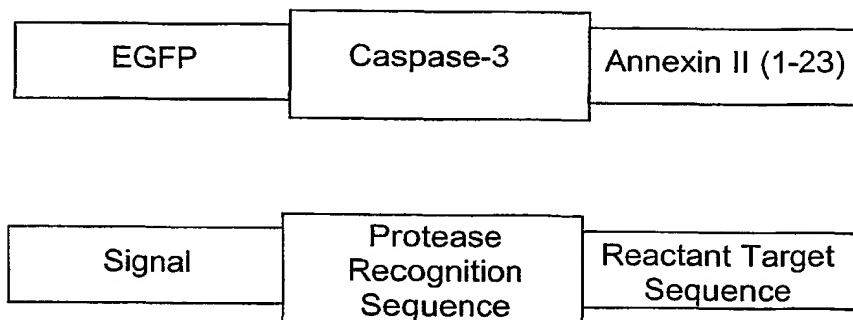
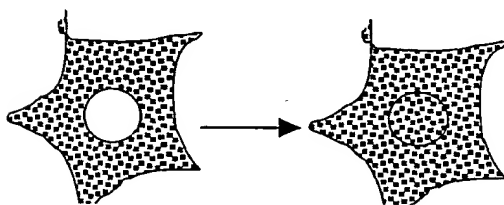
A**B**

Figure 32



BHK cells transfected with DEVD-caspase biosensor. (A) Cells before stimulation of apoptosis. (B) Another field of cells after stimulation with 250 µg/ml cis-platin (4 h).

Figure 33

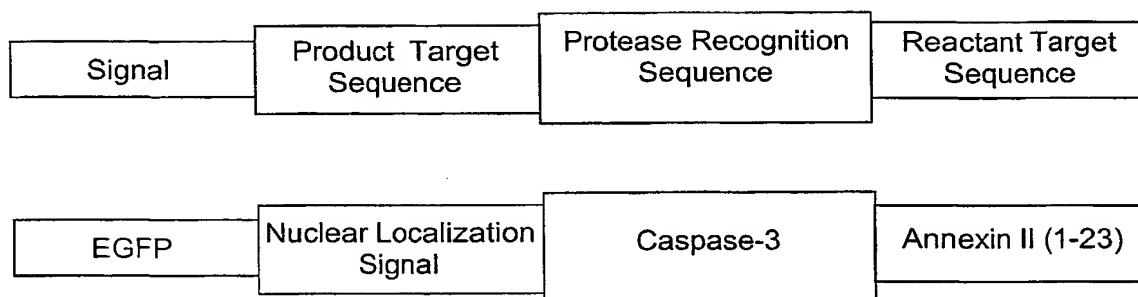


Figure 34

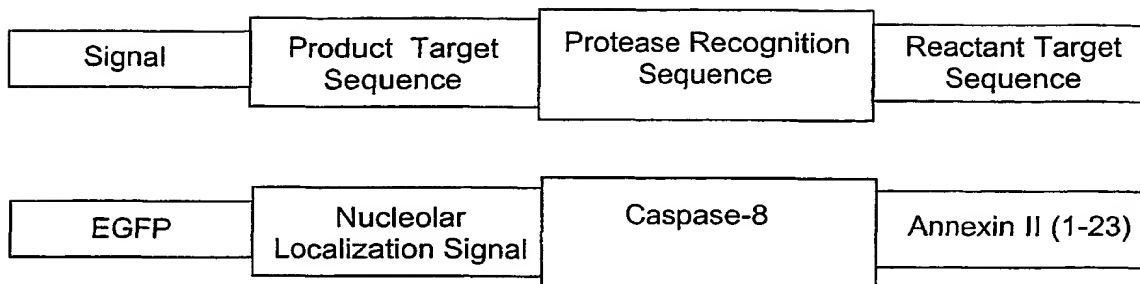


Figure 35

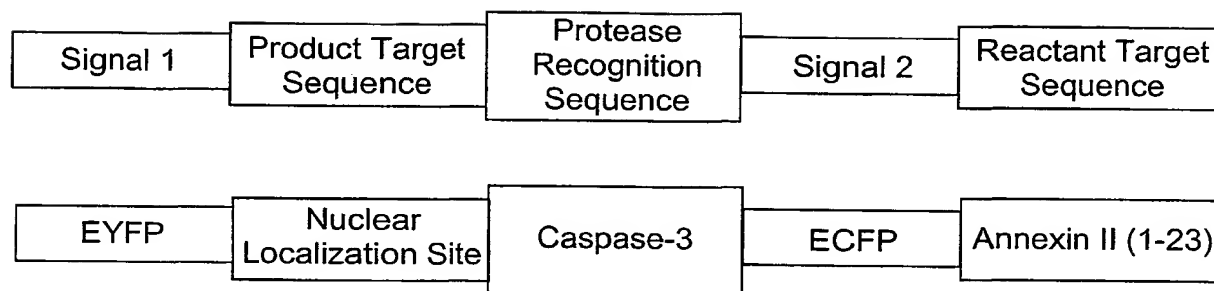
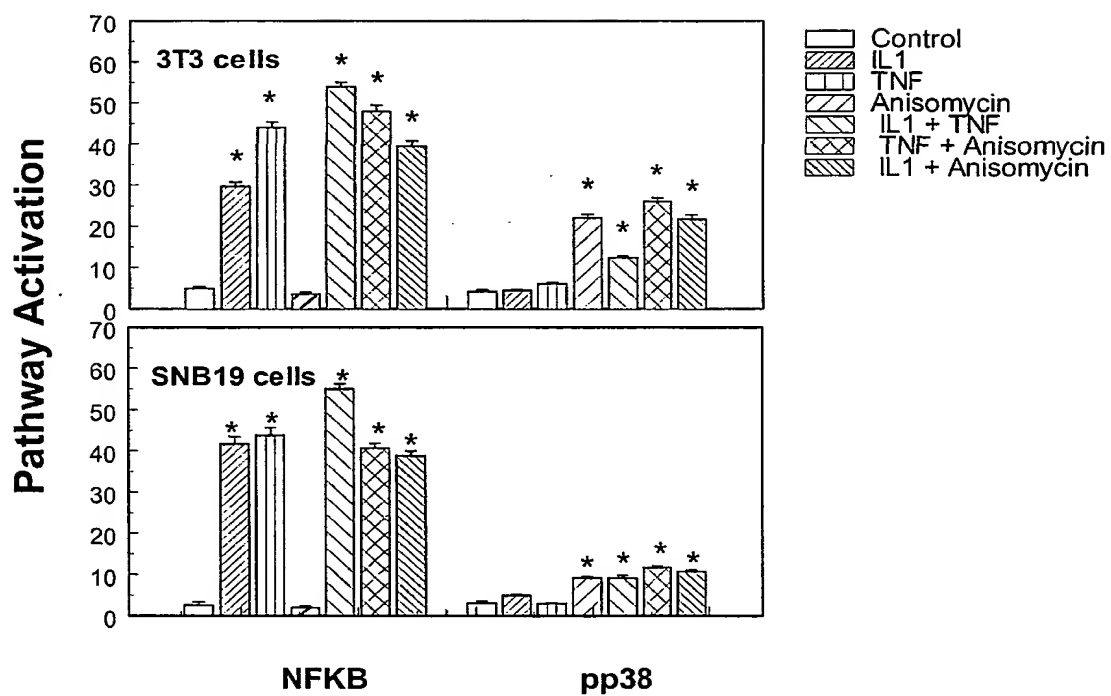


Figure 36



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Kapur, Ravi

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<141> Filed Herewith

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Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
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Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
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Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
			85						90					95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
			115				120						125			

atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac 432
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140

aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac 480
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160

ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc 528
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
 165 170 175

gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc 576
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190

ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg 624
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205

agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc 672
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220

gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc 720
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
 225 230 235 240

gga ctc aga tct ggc gcc ggc gct gga gcc gga gct ggc gcc gga gcc 768
 Gly Leu Arg Ser Gly Ala Gly Ala Gly Ala Gly Ala Gly Ala Gly Ala
 245 250 255

gac gag gtg gac ggc gcc ggc gcc gat gaa gta gat ggc gcc atg tct 816
 Asp Glu Val Asp Gly Ala Gly Ala Asp Glu Val Asp Gly Ala Met Ser
 260 265 270

act gtc cac gaa atc ctg tgc aag ctc agc ttg gag ggt gat cat tct 864
 Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Asp His Ser
 275 280 285

aca ccc cca agt gcc tat tgaatggtga gcaagggcga ggagctgttc 912
 Thr Pro Pro Ser Ala Tyr
 290

accgggggtgg tgcccatcct ggtcgagctg gacggcgacg taaacggcca caagttcagc 972

gtgtccggcg agggcgaggg cgatgccacc tacggcaagc tgaccctgaa gttcatctgc 1032

accaccggca agctgcccgt gccctggccc accctcgtga ccaccctgac ctacggcgtg 1092

cagtgtttca gccgctaccc cgaccacatg aagcagcacg acttcttcaa gtccgccatg 1152

cccgaaggct acgtccagga gcgcaccatc ttcttcaagg acgacggcaa ctacaagacc 1212

cgcgccgagg tgaagttcga gggcgacacc ctggtgaacc gcatcgagct gaagggcatc 1272

gacttcaagg aggacggcaa catcctgggg cacaagctgg agtacaacta caacagccac 1332

aacgtctata tcatggccga caagcagaag aacggcatca aggtgaactt caagatccgc 1392

cacaacatcg aggacggcag cgtgcagctc gccgaccact accagcagaa cccccccatc 1452
 ggcgacggcc ccgtgctgct gcccgacaac cactacctga gcacccagtc cgccctgagc 1512
 aaagacccca acgagaagcg cgatcacatg gtctctgctgg agttcgtgac cgccgccggg 1572
 atcactctcg gcatggacga gctgtacaag tccggactca gatctggcgc cggcgctgga 1632
 gccggagctg gcgccggagc cgacgaggtg gacggcgccg gcgccgatga agtagatggc 1692
 gccatgtcta ctgtccacga aatcctgtgc aagctcagct tggagggtga tcattctaca 1752
 cccccaagtg cctattga 1770

<210> 2

<211> 294

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

GFP-DEV D-Annexin II construct

<400> 2

Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	1	5	10	15
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	20	25	30	
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	35	40	45	
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	50	55	60	
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	65	70	75	80
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	85	90	95	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	100	105	110	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	115	120	125	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	130	135	140	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	145	150	155	160
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	165	170	175	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	180	185	190	

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
 225 230 235 240

Gly Leu Arg Ser Gly Ala Gly Ala Gly Ala Gly Ala Gly Ala Gly Ala
 245 250 255

Asp Glu Val Asp Gly Ala Gly Ala Asp Glu Val Asp Gly Ala Met Ser
 260 265 270

Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Asp His Ser
 275 280 285

Thr Pro Pro Ser Ala Tyr
 290

<210> 3
 <211> 2439
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(2436)

<220>
 <223> Description of Artificial Sequence:
 EYFP-DEVD-MAPKDM construct

<400> 3
 atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg 48
 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15

gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc 96
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30

gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc 144
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45

tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc 192
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60

ttc ggc tac ggc ctg cag tgc ttc gcc cgc tac ccc gac cac atg aag 240
 Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys
 65 70 75 80

cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag 288
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95

cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag 336

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc	528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
ccc gtg ctg ctg ccc gac aac cac tac ctg agc tac cag tcc gcc ctg	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu	
195 200 205	
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag aag	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Lys	
225 230 235 240	
gga gac gaa gtg gac gga gcc gac ctc agt ctt gtg gat gcg ttg aca	768
Gly Asp Glu Val Asp Gly Ala Asp Leu Ser Leu Val Asp Ala Leu Thr	
245 250 255	
gaa cca cct cca gaa att gag gga gaa ata aag cga gac ttc atg gct	816
Glu Pro Pro Pro Glu Ile Glu Gly Glu Ile Lys Arg Asp Phe Met Ala	
260 265 270	
gcg ctg gag gca gag ccc tat gat gac atc gtg gga gaa act gtg gag	864
Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val Gly Glu Thr Val Glu	
275 280 285	
aaa act gag ttt att cct ctc ctg gat ggt gat gag aaa acc ggg aac	912
Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn	
290 295 300	
tca gag tcc aaa aag aaa ccc tgc tta gac act agc cag gtt gaa ggt	960
Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr Ser Gln Val Glu Gly	
305 310 315 320	
atc cca tct tct aaa cca aca ctc cta gcc aat ggt gat cat gga atg	1008
Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn Gly Asp His Gly Met	
325 330 335	
gag ggg aat aac act gca ggg tct cca act gac ttc ctt gaa gag aga	1056
Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg	

340	345	350	
gtg gac tat ccg gat tat cag agc agc cag aac tgg cca gaa gat gca Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala 355 360 365			1104
agc ttt tgt ttc cag cct cag caa gtg tta gat act gac cag gct gag Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp Thr Asp Gln Ala Glu 370 375 380			1152
ccc ttt aac gag cac cgt gat gat ggt ttg gca gat ctg ctc ttt gtc Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe Val 385 390 395 400			1200
tcc agt gga ccc acg aac gct tct gca ttt aca gag cga gac aat cct Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr Glu Arg Asp Asn Pro 405 410 415			1248
tca gaa gac agt tac ggt atg ctt ccc tgt gac tca ttt gct tcc acg Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp Ser Phe Ala Ser Thr 420 425 430			1296
gct gtt gta tct cag gag tgg tct gtg gga gcc cca aac tct cca tgt Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala Pro Asn Ser Pro Cys 435 440 445			1344
tca gag tcc tgt gtc tcc cca gag gtt act ata gaa acc cta cag cca Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile Glu Thr Leu Gln Pro 450 455 460			1392
gca aca gag ctc tcc aag gca gca gaa gtg gaa tca gtg aaa gag cag Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu Ser Val Lys Glu Gln 465 470 475 480			1440
ctg cca gct aaa gca ttg gaa acg atg gca gag cag acc act gat gtg Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu Gln Thr Thr Asp Val 485 490 495			1488
gtg cac tct cca tcc aca gac aca aca cca ggc cca gac aca gag gca Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu Ala 500 505 510			1536
gca ctg gct aaa gac ata gaa gag atc acc aag cca gat gtg ata ttg Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys Pro Asp Val Ile Leu 515 520 525			1584
gca aat gtc acg cag cca tct act gaa tcg gat atg ttc ctg gcc cag Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp Met Phe Leu Ala Gln 530 535 540			1632
gac atg gaa cta ctc aca gga aca gag gca gcc cac gct aac aat atc Asp Met Glu Leu Leu Thr Gly Thr Glu Ala Ala His Ala Asn Asn Ile 545 550 555 560			1680
ata ttg cct aca gaa cca gac gaa tct tca acc aag gat gta gca cca Ile Leu Pro Thr Glu Pro Asp Glu Ser Ser Thr Lys Asp Val Ala Pro 565 570 575			1728
cct atg gaa gaa gaa att gtc cca ggc aat gat acg aca tcc ccc aaa Pro Met Glu Glu Glu Ile Val Pro Gly Asn Asp Thr Thr Ser Pro Lys 580 585 590			1776

gaa aca gag aca aca ctt cca ata aaa atg gac ttg gca cca cct gag Glu Thr Glu Thr Thr Leu Pro Ile Lys Met Asp Leu Ala Pro Pro Glu 595 600 605	1824
gat gtg tta ctt acc aaa gaa aca gaa cta gcc cca gcc aag ggc atg Asp Val Leu Leu Thr Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly Met 610 615 620	1872
gtt tca ctc tca gaa ata gaa gag gct ctg gca aag aat gat gtt cgc Val Ser Leu Ser Glu Ile Glu Glu Ala Leu Ala Lys Asn Asp Val Arg 625 630 635 640	1920
tct gca gaa ata cct gtg gct cag gag aca gtg gtc tca gaa aca gag Ser Ala Glu Ile Pro Val Ala Gln Glu Thr Val Val Ser Glu Thr Glu 645 650 655	1968
gtg gtc ctg gca aca gaa gtg gta ctg ccc tca gat ccc ata aca aca Val Val Leu Ala Thr Glu Val Val Leu Pro Ser Asp Pro Ile Thr Thr 660 665 670	2016
ttg aca aag gat gtg aca ctc ccc tta gaa gca gag aga ccg ttg gtg Leu Thr Lys Asp Val Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu Val 675 680 685	2064
acg gac atg act cca tct ctg gaa aca gaa atg acc cta ggc aaa gag Thr Asp Met Thr Pro Ser Leu Glu Thr Glu Met Thr Leu Gly Lys Glu 690 695 700	2112
aca gct cca ccc aca gaa aca aat ttg ggc atg gcc aaa gac atg tct Thr Ala Pro Pro Thr Glu Thr Asn Leu Gly Met Ala Lys Asp Met Ser 705 710 715 720	2160
cca ctc cca gaa tca gaa gtg act ctg ggc aag gac gtg gtt ata ctt Pro Leu Pro Glu Ser Glu Val Thr Leu Gly Lys Asp Val Val Ile Leu 725 730 735	2208
cca gaa aca aag gtg gct gag ttt aac aat gtg act cca ctt tca gaa Pro Glu Thr Lys Val Ala Glu Phe Asn Asn Val Thr Pro Leu Ser Glu 740 745 750	2256
gaa gag gta acc tca gtc aag gac atg tct ccg tct gca gaa aca gag Glu Glu Val Thr Ser Val Lys Asp Met Ser Pro Ser Ala Glu Thr Glu 755 760 765	2304
gct ccc ctg gct aag aat gct gat ctg cac tca gga aca gag ctg att Ala Pro Leu Ala Lys Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile 770 775 780	2352
gtg gac aac agc atg gct cca gcc tcc gat ctt gca ctg ccc ttg gaa Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu 785 790 795 800	2400
aca aaa gta gca aca gtt cca att aaa gac aaa gga tga Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys Gly 805 810	2439

<210> 4
 <211> 812
 <212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

EYFP-DEVD-MAPKDM construct

<400> 4

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1              5              10              15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
      20              25              30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
      35              40              45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
      50              55              60

Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys
      65              70              75              80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
      85              90              95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
      100             105             110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
      115             120             125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
      130             135             140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
      145             150             155             160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
      165             170             175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
      180             185             190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu
      195             200             205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
      210             215             220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Lys
      225             230             235             240

Gly Asp Glu Val Asp Gly Ala Asp Leu Ser Leu Val Asp Ala Leu Thr
      245             250             255

Glu Pro Pro Pro Glu Ile Glu Gly Glu Ile Lys Arg Asp Phe Met Ala
      260             265             270

Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val Gly Glu Thr Val Glu
      275             280             285

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Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn
 290 295 300
 Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr Ser Gln Val Glu Gly
 305 310 315 320
 Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn Gly Asp His Gly Met
 325 330 335
 Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg
 340 345 350
 Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala
 355 360 365
 Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp Thr Asp Gln Ala Glu
 370 375 380
 Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe Val
 385 390 395 400
 Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr Glu Arg Asp Asn Pro
 405 410 415
 Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp Ser Phe Ala Ser Thr
 420 425 430
 Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala Pro Asn Ser Pro Cys
 435 440 445
 Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile Glu Thr Leu Gln Pro
 450 455 460
 Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu Ser Val Lys Glu Gln
 465 470 475 480
 Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu Gln Thr Thr Asp Val
 485 490 495
 Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu Ala
 500 505 510
 Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys Pro Asp Val Ile Leu
 515 520 525
 Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp Met Phe Leu Ala Gln
 530 535 540
 Asp Met Glu Leu Leu Thr Gly Thr Glu Ala Ala His Ala Asn Asn Ile
 545 550 555 560
 Ile Leu Pro Thr Glu Pro Asp Glu Ser Ser Thr Lys Asp Val Ala Pro
 565 570 575
 Pro Met Glu Glu Glu Ile Val Pro Gly Asn Asp Thr Thr Ser Pro Lys
 580 585 590
 Glu Thr Glu Thr Thr Leu Pro Ile Lys Met Asp Leu Ala Pro Pro Glu
 595 600 605
 Asp Val Leu Leu Thr Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly Met

610	615	620
Val Ser Leu Ser Glu Ile Glu Glu Ala Leu Ala Lys Asn Asp Val Arg 625 630 635 640		
Ser Ala Glu Ile Pro Val Ala Gln Glu Thr Val Val Ser Glu Thr Glu 645 650 655		
Val Val Leu Ala Thr Glu Val Val Leu Pro Ser Asp Pro Ile Thr Thr 660 665 670		
Leu Thr Lys Asp Val Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu Val 675 680 685		
Thr Asp Met Thr Pro Ser Leu Glu Thr Glu Met Thr Leu Gly Lys Glu 690 695 700		
Thr Ala Pro Pro Thr Glu Thr Asn Leu Gly Met Ala Lys Asp Met Ser 705 710 715 720		
Pro Leu Pro Glu Ser Glu Val Thr Leu Gly Lys Asp Val Val Ile Leu 725 730 735		
Pro Glu Thr Lys Val Ala Glu Phe Asn Asn Val Thr Pro Leu Ser Glu 740 745 750		
Glu Glu Val Thr Ser Val Lys Asp Met Ser Pro Ser Ala Glu Thr Glu 755 760 765		
Ala Pro Leu Ala Lys Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile 770 775 780		
Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu 785 790 795 800		
Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys Gly 805 810		

<210> 5

<211> 2439

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1) .. (2436)

<220>

<223> Description of Artificial Sequence:

EYFP-DEAD-MAPKDM construct

<400> 5

atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg	48
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	

gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ttc ggc tac ggc ctg cag tgc ttc gcc cgc tac ccc gac cac atg aag	240
Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag	288
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
cgc acc atc ttc aag gac gac ggc aac tac aag acc cgc gcc gag	336
Arg Thr Ile Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
atc gac ttc aag gag gac ggc aac atc ctg ggc cac aag ctg gag tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc	528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
ccc gtg ctg ctg ccc gac aac cac tac ctg agc tac cag tcc gcc ctg	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu	
195 200 205	
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag ccc	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Pro	
225 230 235 240	
aga gac gaa gcc gac agc gcc gac ctc agt ctt gtg gat gcg ttg aca	768
Arg Asp Glu Ala Asp Ser Ala Asp Leu Ser Leu Val Asp Ala Leu Thr	
245 250 255	
gaa cca cct cca gaa att gag gga gaa ata aag cga gac ttc atg gct	816
Glu Pro Pro Glu Ile Glu Gly Glu Ile Lys Arg Asp Phe Met Ala	
260 265 270	
gcg ctg gag gca gag ccc tat gat gac atc gtg gga gaa act gtg gag	864

Ala	Leu	Glu	Ala	Glu	Pro	Tyr	Asp	Asp	Ile	Val	Gly	Glu	Thr	Val	Glu		
		275					280					285					
aaa	act	gag	ttt	att	cct	ctc	ctg	gat	ggg	gat	gag	aaa	acc	ggg	aac	912	
Lys	Thr	Glu	Phe	Ile	Pro	Leu	Leu	Asp	Gly	Asp	Glu	Lys	Thr	Gly	Asn		
		290				295					300						
tca	gag	tcc	aaa	aag	aaa	ccc	tgc	tta	gac	act	agc	cag	gtt	gaa	ggg	960	
Ser	Glu	Ser	Lys	Lys	Lys	Pro	Cys	Leu	Asp	Thr	Ser	Gln	Val	Glu	Gly		
305					310					315					320		
atc	cca	tct	tct	aaa	cca	aca	ctc	cta	gcc	aat	ggg	gat	cat	gga	atg	1008	
Ile	Pro	Ser	Ser	Lys	Pro	Thr	Leu	Leu	Ala	Asn	Gly	Asp	His	Gly	Met		
				325					330					335			
gag	ggg	aat	aac	act	gca	ggg	tct	cca	act	gac	ttc	ctt	gaa	gag	aga	1056	
Glu	Gly	Asn	Asn	Thr	Ala	Gly	Ser	Pro	Thr	Asp	Phe	Leu	Glu	Glu	Arg		
			340					345					350				
gtg	gac	tat	ccg	gat	tat	cag	agc	agc	cag	aac	tgg	cca	gaa	gat	gca	1104	
Val	Asp	Tyr	Pro	Asp	Tyr	Gln	Ser	Ser	Gln	Asn	Trp	Pro	Glu	Asp	Ala		
		355				360						365					
agc	ttt	tgt	ttc	cag	cct	cag	caa	gtg	tta	gat	act	gac	cag	gct	gag	1152	
Ser	Phe	Cys	Phe	Gln	Pro	Gln	Gln	Val	Leu	Asp	Thr	Asp	Gln	Ala	Glu		
		370				375						380					
ccc	ttt	aac	gag	cac	cgt	gat	gat	ggg	ttg	gca	gat	ctg	ctc	ttt	gtc	1200	
Pro	Phe	Asn	Glu	His	Arg	Asp	Asp	Gly	Leu	Ala	Asp	Leu	Leu	Phe	Val		
385					390					395					400		
tcc	agt	gga	ccc	acg	aac	gct	tct	gca	ttt	aca	gag	cga	gac	aac	cct	1248	
Ser	Ser	Gly	Pro	Thr	Asn	Ala	Ser	Ala	Phe	Thr	Glu	Arg	Asp	Asn	Pro		
				405					410					415			
tca	gaa	gac	agt	tac	ggg	atg	ctt	ccc	tgt	gac	tca	ttt	gct	tcc	acg	1296	
Ser	Glu	Asp	Ser	Tyr	Gly	Met	Leu	Pro	Cys	Asp	Ser	Phe	Ala	Ser	Thr		
			420					425					430				
gct	gtt	gta	tct	cag	gag	tgg	tct	gtg	gga	gcc	cca	aac	tct	cca	tgt	1344	
Ala	Val	Val	Ser	Gln	Glu	Trp	Ser	Val	Gly	Ala	Pro	Asn	Ser	Pro	Cys		
		435					440					445					
tca	gag	tcc	tgt	gtc	tcc	cca	gag	gtt	act	ata	gaa	acc	cta	cag	cca	1392	
Ser	Glu	Ser	Cys	Val	Ser	Pro	Glu	Val	Thr	Ile	Glu	Thr	Leu	Gln	Pro		
		450				455					460						
gca	aca	gag	ctc	tcc	aag	gca	gca	gaa	gtg	gaa	tca	gtg	aaa	gag	cag	1440	
Ala	Thr	Glu	Leu	Ser	Lys	Ala	Ala	Glu	Val	Glu	Ser	Val	Lys	Glu	Gln		
465					470					475					480		
ctg	cca	gct	aaa	gca	ttg	gaa	acg	atg	gca	gag	cag	acc	act	gat	gtg	1488	
Leu	Pro	Ala	Lys	Ala	Leu	Glu	Thr	Met	Ala	Glu	Gln	Thr	Thr	Asp	Val		
				485					490					495			
gtg	cac	tct	cca	tcc	aca	gac	aca	aca	cca	ggc	cca	gac	aca	gag	gca	1536	
Val	His	Ser	Pro	Ser	Thr	Asp	Thr	Thr	Pro	Gly	Pro	Asp	Thr	Glu	Ala		
			500					505					510				
gca	ctg	gct	aaa	gac	ata	gaa	gag	atc	acc	aag	cca	gat	gtg	ata	ttg	1584	
Ala	Leu	Ala	Lys	Asp	Ile	Glu	Glu	Ile	Thr	Lys	Pro	Asp	Val	Ile	Leu		

13

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gct ccc ctg gct aag aat gct gat ctg cac tca gga aca gag ctg att 2352
Ala Pro Leu Ala Lys Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile
770 775 780

gtg gac aac agc atg gct cca gcc tcc gat ctt gca ctg ccc ttg gaa 2400
Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu
785 790 795 800

aca aaa gta gca aca gtt cca att aaa gac aaa gga tga 2439
Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys Gly
805 810

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<210> 6
 <211> 812
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence:
 EYFP-DEAD-MAPKDM construct

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<400> 6
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
50 55 60

Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys
65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu

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195	200	205
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe 210 215 220		
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Pro 225 230 235 240		
Arg Asp Glu Ala Asp Ser Ala Asp Leu Ser Leu Val Asp Ala Leu Thr 245 250 255		
Glu Pro Pro Pro Glu Ile Glu Gly Glu Ile Lys Arg Asp Phe Met Ala 260 265 270		
Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val Gly Glu Thr Val Glu 275 280 285		
Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn 290 295 300		
Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr Ser Gln Val Glu Gly 305 310 315 320		
Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn Gly Asp His Gly Met 325 330 335		
Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg 340 345 350		
Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala 355 360 365		
Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp Thr Asp Gln Ala Glu 370 375 380		
Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe Val 385 390 395 400		
Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr Glu Arg Asp Asn Pro 405 410 415		
Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp Ser Phe Ala Ser Thr 420 425 430		
Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala Pro Asn Ser Pro Cys 435 440 445		
Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile Glu Thr Leu Gln Pro 450 455 460		
Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu Ser Val Lys Glu Gln 465 470 475 480		
Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu Gln Thr Thr Asp Val 485 490 495		
Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu Ala 500 505 510		
Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys Pro Asp Val Ile Leu 515 520 525		

Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp Met Phe Leu Ala Gln
 530 535 540
 Asp Met Glu Leu Leu Thr Gly Thr Glu Ala Ala His Ala Asn Asn Ile
 545 550 555 560
 Ile Leu Pro Thr Glu Pro Asp Glu Ser Ser Thr Lys Asp Val Ala Pro
 565 570 575
 Pro Met Glu Glu Glu Ile Val Pro Gly Asn Asp Thr Thr Ser Pro Lys
 580 585 590
 Glu Thr Glu Thr Thr Leu Pro Ile Lys Met Asp Leu Ala Pro Pro Glu
 595 600 605
 Asp Val Leu Leu Thr Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly Met
 610 615 620
 Val Ser Leu Ser Glu Ile Glu Glu Ala Leu Ala Lys Asn Asp Val Arg
 625 630 635 640
 Ser Ala Glu Ile Pro Val Ala Gln Glu Thr Val Val Ser Glu Thr Glu
 645 650 655
 Val Val Leu Ala Thr Glu Val Val Leu Pro Ser Asp Pro Ile Thr Thr
 660 665 670
 Leu Thr Lys Asp Val Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu Val
 675 680 685
 Thr Asp Met Thr Pro Ser Leu Glu Thr Glu Met Thr Leu Gly Lys Glu
 690 695 700
 Thr Ala Pro Pro Thr Glu Thr Asn Leu Gly Met Ala Lys Asp Met Ser
 705 710 715 720
 Pro Leu Pro Glu Ser Glu Val Thr Leu Gly Lys Asp Val Val Ile Leu
 725 730 735
 Pro Glu Thr Lys Val Ala Glu Phe Asn Asn Val Thr Pro Leu Ser Glu
 740 745 750
 Glu Glu Val Thr Ser Val Lys Asp Met Ser Pro Ser Ala Glu Thr Glu
 755 760 765
 Ala Pro Leu Ala Lys Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile
 770 775 780
 Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu
 785 790 795 800
 Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys Gly
 805 810

<210> 7

<211> 864

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(861)

<220>

<223> Description of Artificial Sequence: F25-MEK1
construct

<400> 7

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt	48
Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
ggt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc	528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195 200 205	


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tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt 672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
210 215 220

gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac acc 720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Thr
225 230 235 240

ggt atg ccc aag aag aag ccg acg ccc atc cag ctg aac ccg gcc ccc 768
Gly Met Pro Lys Lys Lys Pro Thr Pro Ile Gln Leu Asn Pro Ala Pro
245 250 255

gac ggc tct gca gtt aac ggg acc agc tct gcg gag acc aac ttg gag 816
Asp Gly Ser Ala Val Asn Gly Thr Ser Ser Ala Glu Thr Asn Leu Glu
260 265 270

gcc ttg cag aag aag ctg gag gag cta gag ctt gat gag cag cag tga 864
Ala Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Asp Glu Gln Gln
275 280 285

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<210> 8

<211> 287

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: F25-MEK1
construct

<400> 8

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Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
50 55 60

Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65 70 75 80

Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145 150 155 160

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Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
 165 170 175
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220
 Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Thr
 225 230 235 240
 Gly Met Pro Lys Lys Lys Pro Thr Pro Ile Gln Leu Asn Pro Ala Pro
 245 250 255
 Asp Gly Ser Ala Val Asn Gly Thr Ser Ser Ala Glu Thr Asn Leu Glu
 260 265 270
 Ala Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Asp Glu Gln Gln
 275 280 285

<210> 9
 <211> 876
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(873)

<220>
 <223> Description of Artificial Sequence: F25-MEK2
 construct

<400> 9
 atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15
 gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30
 gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45
 tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act 192
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60
 ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa 240
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80
 cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa 288
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu

85										90					95					
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336																			
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu																				
100 105 110																				
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384																			
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly																				
115 120 125																				
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432																			
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr																				
130 135 140																				
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480																			
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn																				
145 150 155 160																				
gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc	528																			
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser																				
165 170 175																				
gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc	576																			
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly																				
180 185 190																				
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt	624																			
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu																				
195 200 205																				
tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt	672																			
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe																				
210 215 220																				
gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac acc	720																			
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Thr																				
225 230 235 240																				
ggg atg ctg gcc cgg agg aag ccg gtg ctg ccg gcg ctc acc atc aac	768																			
Gly Met Leu Ala Arg Arg Lys Pro Val Leu Pro Ala Leu Thr Ile Asn																				
245 250 255																				
cct acc atc gcc gag ggc cca tcc cct acc agc gag ggc gcc tcc gag	816																			
Pro Thr Ile Ala Glu Gly Pro Ser Pro Thr Ser Glu Gly Ala Ser Glu																				
260 265 270																				
gca aac ctg gtg gac ctg cag aag aag ctg gag gag ctg gaa ctt gac	864																			
Ala Asn Leu Val Asp Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Asp																				
275 280 285																				
gag cag cag taa	876																			
Glu Gln Gln																				
290																				

<210> 10

<211> 291

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: F25-MEK2
construct

<400> 10

Met	Ala	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	1	5	10	15
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	20	25	30	
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	35	40	45	
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	50	55	60	
Leu	Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	65	70	75	80
Arg	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	85	90	95	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	100	105	110	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	115	120	125	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	130	135	140	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	145	150	155	160
Gly	Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	165	170	175	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	180	185	190	
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	195	200	205	
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	210	215	220	
Val	Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Asn	Thr	225	230	235	240
Gly	Met	Leu	Ala	Arg	Arg	Lys	Pro	Val	Leu	Pro	Ala	Leu	Thr	Ile	Asn	245	250	255	
Pro	Thr	Ile	Ala	Glu	Gly	Pro	Ser	Pro	Thr	Ser	Glu	Gly	Ala	Ser	Glu	260	265	270	
Ala	Asn	Leu	Val	Asp	Leu	Gln	Lys	Lys	Leu	Glu	Glu	Leu	Glu	Leu	Asp	275	280	285	
Glu	Gln	Gln														290			

<210> 11
 <211> 889
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(888)

<220>
 <223> Description of Artificial Sequence: Caspase
 3-DEVD-substrate construct

<400> 11
 atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15

ggt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30

gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45

tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act 192
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60

ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa 240
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80

cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa 288
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95

agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa 336
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110

gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt 384
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125

att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac 432
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140

aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat 480
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160

gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc 528
 Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
 165 170 175

gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc 576
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly

180	185	190	
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt			624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu			
195	200	205	
tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt			672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe			
210	215	220	
gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc			720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser			
225	230	235	240
gga aga agg aaa cga caa aag cga tcg gct gtt aaa tct gaa gga aag			768
Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Val Lys Ser Glu Gly Lys			
245	250	255	
aga aag tgt gac gaa gtt gat gga att gat gaa gta gca agt act atg			816
Arg Lys Cys Asp Glu Val Asp Gly Ile Asp Glu Val Ala Ser Thr Met			
260	265	270	
tct act gtc cac gaa atc ctg tgc aag ctc agc ttg gag ggt gtt cat			864
Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Val His			
275	280	285	
tct aca ccc cca agt acc cgg atc c			889
Ser Thr Pro Pro Ser Thr Arg Ile			
290	295		

<210> 12

<211> 296

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase
3-DEVD-substrate construct

<400> 12

Met	Ala	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu
1				5					10					15	

Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly
			20					25					30		

Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile
		35					40					45			

Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr
	50					55					60				

Leu	Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys
65					70					75					80

Arg	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu
			85						90					95	

Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu
			100					105						110	

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
 165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220

Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser
 225 230 235 240

Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Val Lys Ser Glu Gly Lys
 245 250 255

Arg Lys Cys Asp Glu Val Asp Gly Ile Asp Glu Val Ala Ser Thr Met
 260 265 270

Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Val His
 275 280 285

Ser Thr Pro Pro Ser Thr Arg Ile
 290 295

<210> 13

<211> 846

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1) .. (846)

<220>

<223> Description of Artificial Sequence: Caspase
 6-VEID-substrate construct

<400> 13

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15

gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30

gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144

Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile		
		35					40					45					
tgc	act	act	ggc	aaa	ctg	cct	ggt	cca	tgg	cca	aca	cta	gtc	act	act	192	
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr		
	50					55					60						
ctg	tgc	tat	ggg	gtt	caa	tgc	ttt	tca	aga	tac	ccg	gat	cat	atg	aaa	240	
Leu	Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys		
	65				70					75					80		
cgg	cat	gac	ttt	ttc	aag	agt	gcc	atg	ccc	gaa	ggg	tat	gta	cag	gaa	288	
Arg	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu		
				85					90						95		
agg	acc	atc	ttc	ttc	aaa	gat	gac	ggc	aac	tac	aag	aca	cgt	gct	gaa	336	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu		
			100					105					110				
gtc	aag	ttt	gaa	ggg	gat	acc	ctt	gtt	aat	aga	atc	gag	tta	aaa	ggg	384	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly		
		115					120					125					
att	gac	ttc	aag	gaa	gat	ggc	aac	att	ctg	gga	cac	aaa	ttg	gaa	tac	432	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr		
	130					135					140						
aac	tat	aac	tca	cac	aat	gta	tac	atc	atg	gca	gac	aaa	caa	aag	aat	480	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn		
	145				150					155					160		
gga	atc	aaa	gtg	aac	ttc	aag	acc	cgc	cac	aac	att	gaa	gat	gga	agc	528	
Gly	Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser		
				165					170					175			
gtt	caa	cta	gca	gac	cat	tat	caa	caa	aat	act	cca	att	ggc	gat	ggc	576	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly		
			180					185					190				
cct	gtc	ctt	tta	cca	gac	aac	cat	tac	ctg	tcc	aca	caa	tct	gcc	ctt	624	
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu		
		195					200					205					
tcg	aaa	gat	ccc	aac	gaa	aag	aga	gac	cac	atg	gtc	ctt	ctt	gag	ttt	672	
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe		
	210					215					220						
gta	aca	gct	gct	ggg	att	aca	cat	ggc	atg	gat	gaa	ctg	tac	aac	tcc	720	
Val	Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Asn	Ser		
	225				230					235					240		
gga	aga	agg	aaa	cga	caa	aag	cga	tcg	aca	aga	ctt	gtt	gaa	att	gac	768	
Gly	Arg	Arg	Lys	Arg	Gln	Lys	Arg	Ser	Thr	Arg	Leu	Val	Glu	Ile	Asp		
				245					250					255			
aac	agt	act	atg	agc	aca	gta	cac	gaa	att	tta	tgt	aaa	tta	agc	tta	816	
Asn	Ser	Thr	Met	Ser	Thr	Val	His	Glu	Ile	Leu	Cys	Lys	Leu	Ser	Leu		
			260					265					270				
gaa	gga	gta	cac	agt	aca	cca	cca	agc	gca							846	
Glu	Gly	Val	His	Ser	Thr	Pro	Pro	Ser	Ala								

275

280

<210> 14

<211> 282

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase
6-VEID-substrate construct

<400> 14

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Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
  1           5           10           15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
          20           25           30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
      35           40           45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
  50           55           60

Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
  65           70           75           80

Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
          85           90           95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
      100           105           110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
      115           120           125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
      130           135           140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
      145           150           155           160

Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
          165           170           175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
      180           185           190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
      195           200           205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
      210           215           220

Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser
      225           230           235           240

Gly Arg Arg Lys Arg Gln Lys Arg Ser Thr Arg Leu Val Glu Ile Asp
          245           250           255

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Asn Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu
 260 265 270

Glu Gly Val His Ser Thr Pro Pro Ser Ala
 275 280

<210> 15

<211> 876

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(876)

<220>

<223> Description of Artificial Sequence: Caspase 8-VETD
 construct

<400> 15

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15

gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30

gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45

tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act 192
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60

ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa 240
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80

cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa 288
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95

agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa 336
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110

gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt 384
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125

att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac 432
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140

aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat 480
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160

gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc 528
 Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
 165 170 175

gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc 576
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190

cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt 624
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205

tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt 672
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220

gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc 720
 Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser
 225 230 235 240

gga aga agc aaa cga caa aag cga tcg tat gaa aaa gga ata cca gtt 768
 Gly Arg Ser Lys Arg Gln Lys Arg Ser Tyr Glu Lys Gly Ile Pro Val
 245 250 255

gaa aca gac agc gaa gag caa gct tat agt act atg tct act gtc cac 816
 Glu Thr Asp Ser Glu Glu Gln Ala Tyr Ser Thr Met Ser Thr Val His
 260 265 270

gaa atc ctg tgc aag ctc agc ttg gag ggt gtt cat tct aca ccc cca 864
 Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Val His Ser Thr Pro Pro
 275 280 285

agt gcc gga tcc 876
 Ser Ala Gly Ser
 290

<210> 16

<211> 292

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase 8-VETD construct

<400> 16

Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60

Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80

<400> 17
atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48
Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1 5 10 15

ggt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc	528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195 200 205	
tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc	720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser	
225 230 235 240	
gga aga agg aaa cga caa aag cga tcg gca ggt gac gaa gtt gat gca	768
Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Asp Glu Val Asp Ala	
245 250 255	

ggt gac gaa gtt gat gca ggt gac gaa gtt gat gca ggt gac gaa gtt 816
 Gly Asp Glu Val Asp Ala Gly Asp Glu Val Asp Ala Gly Asp Glu Val
 260 265 270

gac gca ggt agt act atg tct act gtc cac gaa atc ctg tgc aag ctc 864
 Asp Ala Gly Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu
 275 280 285

agc ttg gag ggt gtt cat tct aca ccc cca agt gcc gga tcc 906
 Ser Leu Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser
 290 295 300

<210> 18

<211> 302

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Cas 3-multiple
 DEVD construct

<400> 18

Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60

Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80

Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
 165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220

Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser
 225 230 235 240

Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Asp Glu Val Asp Ala
 245 250 255

Gly Asp Glu Val Asp Ala Gly Asp Glu Val Asp Ala Gly Asp Glu Val
 260 265 270

Asp Ala Gly Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu
 275 280 285

Ser Leu Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser
 290 295 300

<210> 19
 <211> 906
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(885)

<220>
 <223> Description of Artificial Sequence: Caspase
 8-multiple VETD construct

<400> 19
 atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15

gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30

gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45

tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act 192
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60

ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa 240
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80

cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa 288
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95

agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa 336
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110

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gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt 384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
      115                      120                      125

att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac 432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
      130                      135                      140

aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat 480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
      145                      150                      155                      160

gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc 528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
      165                      170                      175

gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc 576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
      180                      185                      190

cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt 624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
      195                      200                      205

tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt 672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
      210                      215                      220

gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc 720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser
      225                      230                      235                      240

gga aga agg aaa cga caa aag cga tcg gca ggt gtt gaa aca gac gca 768
Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Val Glu Thr Asp Ala
      245                      250                      255

ggg gtt gaa aca gac gca ggt gtt gaa aca gac gca ggt gtt gaa aca 816
Gly Val Glu Thr Asp Ala Gly Val Glu Thr Asp Ala Gly Val Glu Thr
      260                      265                      270

gac gca ggt agt act atg tct act gtc cac gaa atc ctg tgc aag ctc 864
Asp Ala Gly Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu
      275                      280                      285

agc ttg gag ggt gtt cat tct acacccccaa gtgccggatc c 906
Ser Leu Glu Gly Val His Ser
      290                      295

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<210> 20

<211> 295

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase
8-multiple VETD construct

<400> 20

Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu

1	5	10	15
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	20	25	30
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	35	40	45
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	50	55	60
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	65	70	75
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	85	90	95
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	100	105	110
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	115	120	125
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	130	135	140
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	145	150	155
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	165	170	175
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	180	185	190
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	195	200	205
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	210	215	220
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser	225	230	235
Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Val Glu Thr Asp Ala	245	250	255
Gly Val Glu Thr Asp Ala Gly Val Glu Thr Asp Ala Gly Val Glu Thr	260	265	270
Asp Ala Gly Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu	275	280	285
Ser Leu Glu Gly Val His Ser	290	295	

<210> 21

<211> 4833

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(4830)

<220>

<223> Description of Artificial Sequence:

EYFP-DEVD-MAP4-EBFP construct

<400> 21

atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	gtg	gtg	ccc	atc	ctg	48
Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5					10					15		
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
			35				40					45				
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55					60					
ttc	ggc	tac	ggc	ctg	cag	tgc	ttc	gcc	cgc	tac	ccc	gac	cac	atg	aag	240
Phe	Gly	Tyr	Gly	Leu	Gln	Cys	Phe	Ala	Arg	Tyr	Pro	Asp	His	Met	Lys	
65				70					75						80	
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
			85						90					95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
		115					120					125				
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
	130					135					140					
aac	tac	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	
145					150					155					160	
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
			165					170					175			
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180					185					190			
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	tac	cag	tcc	gcc	ctg	624
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Tyr	Gln	Ser	Ala	Leu	
		195					200					205				

agc	aaa	gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	672
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	
210						215					220					
gtg	acc	gcc	gcc	ggg	atc	act	ctc	ggc	atg	gac	gag	ctg	tac	aag	aag	720
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Lys	
225					230					235					240	
gga	gac	gaa	gtg	gac	gga	atg	gcc	gac	ctc	agt	ctt	gtg	gat	gcg	ttg	768
Gly	Asp	Glu	Val	Asp	Gly	Met	Ala	Asp	Leu	Ser	Leu	Val	Asp	Ala	Leu	
				245					250					255		
aca	gaa	cca	cct	cca	gaa	att	gag	gga	gaa	ata	aag	cga	gac	ttc	atg	816
Thr	Glu	Pro	Pro	Pro	Glu	Ile	Glu	Gly	Glu	Ile	Lys	Arg	Asp	Phe	Met	
			260					265						270		
gct	gcg	ctg	gag	gca	gag	ccc	tat	gat	gac	atc	gtg	gga	gaa	act	gtg	864
Ala	Ala	Leu	Glu	Ala	Glu	Pro	Tyr	Asp	Asp	Ile	Val	Gly	Glu	Thr	Val	
		275					280					285				
gag	aaa	act	gag	ttt	att	cct	ctc	ctg	gat	ggc	gat	gag	aaa	acc	ggg	912
Glu	Lys	Thr	Glu	Phe	Ile	Pro	Leu	Leu	Asp	Gly	Asp	Glu	Lys	Thr	Gly	
	290					295					300					
aac	tca	gag	tcc	aaa	aag	aaa	ccc	tgc	tta	gac	act	agc	cag	gtt	gaa	960
Asn	Ser	Glu	Ser	Lys	Lys	Lys	Pro	Cys	Leu	Asp	Thr	Ser	Gln	Val	Glu	
305					310					315					320	
ggc	atc	cca	tct	tct	aaa	cca	aca	ctc	cta	gcc	aat	ggc	gat	cat	gga	1008
Gly	Ile	Pro	Ser	Ser	Lys	Pro	Thr	Leu	Leu	Ala	Asn	Gly	Asp	His	Gly	
				325					330					335		
atg	gag	ggg	aat	aac	act	gca	ggg	tct	cca	act	gac	ttc	ctt	gaa	gag	1056
Met	Glu	Gly	Asn	Asn	Thr	Ala	Gly	Ser	Pro	Thr	Asp	Phe	Leu	Glu	Glu	
			340					345					350			
aga	gtg	gac	tat	cgc	gat	tat	cag	agc	agc	cag	aac	tgg	cca	gaa	gat	1104
Arg	Val	Asp	Tyr	Pro	Asp	Tyr	Gln	Ser	Ser	Gln	Asn	Trp	Pro	Glu	Asp	
		355					360					365				
gca	agc	ttt	tgt	ttc	cag	cct	cag	caa	gtg	tta	gat	act	gac	cag	gct	1152
Ala	Ser	Phe	Cys	Phe	Gln	Pro	Gln	Gln	Val	Leu	Asp	Thr	Asp	Gln	Ala	
		370				375					380					
gag	ccc	ttt	aac	gag	cac	cgt	gat	gat	ggc	ttg	gca	gat	ctg	ctc	ttt	1200
Glu	Pro	Phe	Asn	Glu	His	Arg	Asp	Asp	Gly	Leu	Ala	Asp	Leu	Leu	Phe	
385					390					395					400	
gtc	tcc	agt	gga	ccc	acg	aac	gct	tct	gca	ttt	aca	gag	cga	gac	aat	1248
Val	Ser	Ser	Gly	Pro	Thr	Asn	Ala	Ser	Ala	Phe	Thr	Glu	Arg	Asp	Asn	
				405					410					415		
cct	tca	gaa	gac	agt	tac	ggc	atg	ctt	ccc	tgt	gac	tca	ttt	gct	tcc	1296
Pro	Ser	Glu	Asp	Ser	Tyr	Gly	Met	Leu	Pro	Cys	Asp	Ser	Phe	Ala	Ser	
			420					425					430			
acg	gct	gtt	gta	tct	cag	gag	tgg	tct	gtg	gga	gcc	cca	aac	tct	cca	1344
Thr	Ala	Val	Val	Ser	Gln	Glu	Trp	Ser	Val	Gly	Ala	Pro	Asn	Ser	Pro	
		435					440					445				

tgt tca gag tcc tgt gtc tcc cca gag gtt act ata gaa acc cta cag	1392
Cys Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile Glu Thr Leu Gln	
450 455 460	
cca gca aca gag ctc tcc aag gca gca gaa gtg gaa tca gtg aaa gag	1440
Pro Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu Ser Val Lys Glu	
465 470 475 480	
cag ctg cca gct aaa gca ttg gaa acg atg gca gag cag acc act gat	1488
Gln Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu Gln Thr Thr Asp	
485 490 495	
gtg gtg cac tct cca tcc aca gac aca aca cca ggc cca gac aca gag	1536
Val Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu	
500 505 510	
gca gca ctg gct aaa gac ata gaa gag atc acc aag cca gat gtg ata	1584
Ala Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys Pro Asp Val Ile	
515 520 525	
ttg gca aat gtc acg cag cca tct act gaa tcg gat atg ttc ctg gcc	1632
Leu Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp Met Phe Leu Ala	
530 535 540	
cag gac atg gaa cta ctc aca gga aca gag gca gcc cac gct aac aat	1680
Gln Asp Met Glu Leu Leu Thr Gly Thr Glu Ala Ala His Ala Asn Asn	
545 550 555 560	
atc ata ttg cct aca gaa cca gac gaa tct tca acc aag gat gta gca	1728
Ile Ile Leu Pro Thr Glu Pro Asp Glu Ser Ser Thr Lys Asp Val Ala	
565 570 575	
cca cct atg gaa gaa gaa att gtc cca ggc aat gat acg aca tcc ccc	1776
Pro Pro Met Glu Glu Glu Ile Val Pro Gly Asn Asp Thr Thr Ser Pro	
580 585 590	
aaa gaa aca gag aca aca ctt cca ata aaa atg gac ttg gca cca cct	1824
Lys Glu Thr Glu Thr Thr Leu Pro Ile Lys Met Asp Leu Ala Pro Pro	
595 600 605	
gag gat gtg tta ctt acc aaa gaa aca gaa cta gcc cca gcc aag ggc	1872
Glu Asp Val Leu Leu Thr Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly	
610 615 620	
atg gtt tca ctc tca gaa ata gaa gag gct ctg gca aag aat gat gtt	1920
Met Val Ser Leu Ser Glu Ile Glu Glu Ala Leu Ala Lys Asn Asp Val	
625 630 635 640	
cgc tct gca gaa ata cct gtg gct cag gag aca gtg gtc tca gaa aca	1968
Arg Ser Ala Glu Ile Pro Val Ala Gln Glu Thr Val Val Ser Glu Thr	
645 650 655	
gag gtg gtc ctg gca aca gaa gtg gta ctg ccc tca gat ccc ata aca	2016
Glu Val Val Leu Ala Thr Glu Val Leu Pro Ser Asp Pro Ile Thr	
660 665 670	
aca ttg aca aag gat gtg aca ctc ccc tta-gaa gca gag aga ccg ttg	2064
Thr Leu Thr Lys Asp Val Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu	
675 680 685	
gtg acg gac atg act cca tct ctg gaa aca gaa atg acc cta ggc aaa	2112

Val	Thr	Asp	Met	Thr	Pro	Ser	Leu	Glu	Thr	Glu	Met	Thr	Leu	Gly	Lys		
690						695					700						
gag	aca	gct	cca	ccc	aca	gaa	aca	aat	ttg	ggc	atg	gcc	aaa	gac	atg	2160	
Glu	Thr	Ala	Pro	Pro	Thr	Glu	Thr	Asn	Leu	Gly	Met	Ala	Lys	Asp	Met		
705					710				715						720		
tct	cca	ctc	cca	gaa	tca	gaa	gtg	act	ctg	ggc	aag	gac	gtg	gtt	ata	2208	
Ser	Pro	Leu	Pro	Glu	Ser	Glu	Val	Thr	Leu	Gly	Lys	Asp	Val	Val	Ile		
				725					730						735		
ctt	cca	gaa	aca	aag	gtg	gct	gag	ttt	aac	aat	gtg	act	cca	ctt	tca	2256	
Leu	Pro	Glu	Thr	Lys	Val	Ala	Glu	Phe	Asn	Asn	Val	Thr	Pro	Leu	Ser		
			740					745							750		
gaa	gaa	gag	gta	acc	tca	gtc	aag	gac	atg	tct	ccg	tct	gca	gaa	aca	2304	
Glu	Glu	Glu	Val	Thr	Ser	Val	Lys	Asp	Met	Ser	Pro	Ser	Ala	Glu	Thr		
		755					760					765					
gag	gct	ccc	ctg	gct	aag	aat	gct	gat	ctg	cac	tca	gga	aca	gag	ctg	2352	
Glu	Ala	Pro	Leu	Ala	Lys	Asn	Ala	Asp	Leu	His	Ser	Gly	Thr	Glu	Leu		
	770					775						780					
att	gtg	gac	aac	agc	atg	gct	cca	gcc	tcc	gat	ctt	gca	ctg	ccc	ttg	2400	
Ile	Val	Asp	Asn	Ser	Met	Ala	Pro	Ala	Ser	Asp	Leu	Ala	Leu	Pro	Leu		
785					790					795					800		
gaa	aca	aaa	gta	gca	aca	gtt	cca	att	aaa	gac	aaa	gga	act	gta	cag	2448	
Glu	Thr	Lys	Val	Ala	Thr	Val	Pro	Ile	Lys	Asp	Lys	Gly	Thr	Val	Gln		
				805					810						815		
act	gaa	gaa	aaa	cca	cgt	gaa	gac	tcc	cag	tta	gca	tct	atg	cag	cac	2496	
Thr	Glu	Glu	Lys	Pro	Arg	Glu	Asp	Ser	Gln	Leu	Ala	Ser	Met	Gln	His		
			820					825						830			
aag	gga	cag	tca	aca	gta	cct	cct	tgc	acg	gct	tca	cca	gaa	cca	gtc	2544	
Lys	Gly	Gln	Ser	Thr	Val	Pro	Pro	Cys	Thr	Ala	Ser	Pro	Glu	Pro	Val		
		835					840					845					
aaa	gct	gca	gaa	caa	atg	tct	acc	tta	cca	ata	gat	gca	cct	tct	cca	2592	
Lys	Ala	Ala	Glu	Gln	Met	Ser	Thr	Leu	Pro	Ile	Asp	Ala	Pro	Ser	Pro		
	850					855					860						
tta	gag	aac	tta	gag	cag	aag	gaa	acg	cct	ggc	agc	cag	cct	tct	gag	2640	
Leu	Glu	Asn	Leu	Glu	Gln	Lys	Glu	Thr	Pro	Gly	Ser	Gln	Pro	Ser	Glu		
865					870					875					880		
cct	tgc	tca	gga	gta	tcc	cgg	caa	gaa	gaa	gca	aag	gct	gct	gta	ggt	2688	
Pro	Cys	Ser	Gly	Val	Ser	Arg	Gln	Glu	Glu	Ala	Lys	Ala	Ala	Val	Gly		
				885					890						895		
gtg	act	gga	aat	gac	atc	act	acc	ccg	cca	aac	aag	gag	cca	cca	cca	2736	
Val	Thr	Gly	Asn	Asp	Ile	Thr	Thr	Pro	Pro	Asn	Lys	Glu	Pro	Pro	Pro		
			900					905							910		
agc	cca	gaa	aag	aaa	gca	aag	cct	ttg	gcc	acc	act	caa	cct	gca	aag	2784	
Ser	Pro	Glu	Lys	Lys	Ala	Lys	Pro	Leu	Ala	Thr	Thr	Gln	Pro	Ala	Lys		
		915					920					925					
act	tca	aca	tcg	aaa	gcc	aaa	aca	cag	ccc	act	tct	ctc	cct	aag	caa	2832	
Thr	Ser	Thr	Ser	Lys	Ala	Lys	Thr	Gln	Pro	Thr	Ser	Leu	Pro	Lys	Gln		

930	935	940	
cca gct ccc acc acc tct ggt ggg ttg aat aaa aaa ccc atg agc ctc			2880
Pro Ala Pro Thr Thr Ser Gly Gly Leu Asn Lys Lys Pro Met Ser Leu			
945	950	955	960
gcc tca ggc tca gtg cca gct gcc cca cac aaa cgc cct gct gct gcc			2928
Ala Ser Gly Ser Val Pro Ala Ala Pro His Lys Arg Pro Ala Ala Ala			
	965	970	975
act gct act gcc agg cct tcc acc cta cct gcc aga gac gtg aag cca			2976
Thr Ala Thr Ala Arg Pro Ser Thr Leu Pro Ala Arg Asp Val Lys Pro			
	980	985	990
aag cca att aca gaa gct aag gtt gcc gaa aag cgg acc tct cca tcc			3024
Lys Pro Ile Thr Glu Ala Lys Val Ala Glu Lys Arg Thr Ser Pro Ser			
	995	1000	1005
aag cct tca tct gcc cca gcc ctc aaa cct gga cct aaa acc acc cca			3072
Lys Pro Ser Ser Ala Pro Ala Leu Lys Pro Gly Pro Lys Thr Thr Pro			
	1010	1015	1020
acc gtt tca aaa gcc aca tct ccc tca act ctt gtt tcc act gga cca			3120
Thr Val Ser Lys Ala Thr Ser Pro Ser Thr Leu Val Ser Thr Gly Pro			
	1025	1030	1035
agt agt aga agt cca gct aca act ctg cct aag agg cca acc agc atc			3168
Ser Ser Arg Ser Pro Ala Thr Thr Leu Pro Lys Arg Pro Thr Ser Ile			
	1045	1050	1055
aag act gag ggg aaa cct gct gat gtc aaa agg atg act gct aag tct			3216
Lys Thr Glu Gly Lys Pro Ala Asp Val Lys Arg Met Thr Ala Lys Ser			
	1060	1065	1070
gcc tca gct gac ttg agt cgc tca aag acc acc tct gcc agt tct gtg			3264
Ala Ser Ala Asp Leu Ser Arg Ser Lys Thr Thr Ser Ala Ser Ser Val			
	1075	1080	1085
aag aga aac acc act ccc act ggg gca gca ccc cca gca ggg atg act			3312
Lys Arg Asn Thr Thr Pro Thr Gly Ala Ala Pro Pro Ala Gly Met Thr			
	1090	1095	1100
tcc act cga gtc aag ccc atg tct gca cct agc cgc tct tct ggg gct			3360
Ser Thr Arg Val Lys Pro Met Ser Ala Pro Ser Arg Ser Ser Gly Ala			
	1105	1110	1115
ctt tct gtg gac aag aag ccc act tcc act aag cct agc tcc tct gct			3408
Leu Ser Val Asp Lys Lys Pro Thr Ser Thr Lys Pro Ser Ser Ser Ala			
	1125	1130	1135
ccc agg gtg agc cgc ctg gcc aca act gtt tct gcc cct gac ctg aag			3456
Pro Arg Val Ser Arg Leu Ala Thr Thr Val Ser Ala Pro Asp Leu Lys			
	1140	1145	1150
agt gtt cgc tcc aag gtc ggc tct aca gaa aac atc aaa cac cag cct			3504
Ser Val Arg Ser Lys Val Gly Ser Thr Glu Asn Ile Lys His Gln Pro			
	1155	1160	1165
gga gga ggc cgg gcc aaa gta gag aaa aaa aca gag gca gct acc aca			3552
Gly Gly Gly Arg Ala Lys Val Glu Lys Lys Thr Glu Ala Ala Thr Thr			
	1170	1175	1180

40

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ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc ctg acc cac ggc gtg 4320
Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Thr His Gly Val
1425          1430          1435          1440

cag tgc ttc agc cgc tac ccc gac cac atg aag cag cac gac ttc ttc 4368
Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His Asp Phe Phe
          1445          1450          1455

aag tcc gcc atg ccc gaa ggc tac gtc cag gag cgc acc atc ttc ttc 4416
Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe
          1460          1465          1470

aag gac gac ggc aac tac aag acc cgc gcc gag gtg aag ttc gag ggc 4464
Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly
          1475          1480          1485

gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc aag gag 4512
Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu
          1490          1495          1500

gac ggc aac atc ctg ggg cac aag ctg gag tac aac ttc aac agc cac 4560
Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Phe Asn Ser His
1505          1510          1515          1520

aac gtc tat atc atg gcc gac aag cag aag aac ggc atc aag gtg aac 4608
Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn
          1525          1530          1535

ttc aag atc cgc cac aac atc gag gac ggc agc gtg cag ctc gcc gac 4656
Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp
          1540          1545          1550

cac tac cag cag aac acc ccc atc ggc gac ggc ccc gtg ctg ctg ccc 4704
His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro
          1555          1560          1565

gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa gac ccc aac 4752
Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn
          1570          1575          1580

gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg 4800
Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly
1585          1590          1595          1600

atc act ctc ggc atg gac gag ctg tac aag tag 4833
Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
          1605          1610

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<210> 22

<211> 1610

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
EYFP-DEVD-MAP4-EBFP construct

<400> 22

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
  1              5              10              15

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Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
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 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60
 Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys
 65 70 75 80
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
 165 170 175
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu
 195 200 205
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Lys
 225 230 235 240
 Gly Asp Glu Val Asp Gly Met Ala Asp Leu Ser Leu Val Asp Ala Leu
 245 250 255
 Thr Glu Pro Pro Pro Glu Ile Glu Gly Glu Ile Lys Arg Asp Phe Met
 260 265 270
 Ala Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val Gly Glu Thr Val
 275 280 285
 Glu Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly
 290 295 300
 Asn Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr Ser Gln Val Glu
 305 310 315 320
 Gly Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn Gly Asp His Gly
 325 330 335

Met Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu
 340 345 350
 Arg Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp
 355 360 365
 Ala Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp Thr Asp Gln Ala
 370 375 380
 Glu Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe
 385 390 395 400
 Val Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr Glu Arg Asp Asn
 405 410 415
 Pro Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp Ser Phe Ala Ser
 420 425 430
 Thr Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala Pro Asn Ser Pro
 435 440 445
 Cys Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile Glu Thr Leu Gln
 450 455 460
 Pro Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu Ser Val Lys Glu
 465 470 475 480
 Gln Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu Gln Thr Thr Asp
 485 490 495
 Val Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu
 500 505 510
 Ala Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys Pro Asp Val Ile
 515 520 525
 Leu Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp Met Phe Leu Ala
 530 535 540
 Gln Asp Met Glu Leu Leu Thr Gly Thr Glu Ala Ala His Ala Asn Asn
 545 550 555 560
 Ile Ile Leu Pro Thr Glu Pro Asp Glu Ser Ser Thr Lys Asp Val Ala
 565 570 575
 Pro Pro Met Glu Glu Glu Ile Val Pro Gly Asn Asp Thr Thr Ser Pro
 580 585 590
 Lys Glu Thr Glu Thr Thr Leu Pro Ile Lys Met Asp Leu Ala Pro Pro
 595 600 605
 Glu Asp Val Leu Leu Thr Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly
 610 615 620
 Met Val Ser Leu Ser Glu Ile Glu Glu Ala Leu Ala Lys Asn Asp Val
 625 630 635 640
 Arg Ser Ala Glu Ile Pro Val Ala Gln Glu Thr Val Val Ser Glu Thr
 645 650 655
 Glu Val Val Leu Ala Thr Glu Val Val Leu Pro Ser Asp Pro Ile Thr

660					665					670					
Thr	Leu	Thr	Lys	Asp	Val	Thr	Leu	Pro	Leu	Glu	Ala	Glu	Arg	Pro	Leu
	675						680						685		
Val	Thr	Asp	Met	Thr	Pro	Ser	Leu	Glu	Thr	Glu	Met	Thr	Leu	Gly	Lys
	690						695						700		
Glu	Thr	Ala	Pro	Pro	Thr	Glu	Thr	Asn	Leu	Gly	Met	Ala	Lys	Asp	Met
	705						710						715		
Ser	Pro	Leu	Pro	Glu	Ser	Glu	Val	Thr	Leu	Gly	Lys	Asp	Val	Val	Ile
				725					730					735	
Leu	Pro	Glu	Thr	Lys	Val	Ala	Glu	Phe	Asn	Asn	Val	Thr	Pro	Leu	Ser
				740					745					750	
Glu	Glu	Glu	Val	Thr	Ser	Val	Lys	Asp	Met	Ser	Pro	Ser	Ala	Glu	Thr
			755						760					765	
Glu	Ala	Pro	Leu	Ala	Lys	Asn	Ala	Asp	Leu	His	Ser	Gly	Thr	Glu	Leu
	770								775					780	
Ile	Val	Asp	Asn	Ser	Met	Ala	Pro	Ala	Ser	Asp	Leu	Ala	Leu	Pro	Leu
	785														800
Glu	Thr	Lys	Val	Ala	Thr	Val	Pro	Ile	Lys	Asp	Lys	Gly	Thr	Val	Gln
				805											815
Thr	Glu	Glu	Lys	Pro	Arg	Glu	Asp	Ser	Gln	Leu	Ala	Ser	Met	Gln	His
				820										830	
Lys	Gly	Gln	Ser	Thr	Val	Pro	Pro	Cys	Thr	Ala	Ser	Pro	Glu	Pro	Val
														845	
Lys	Ala	Ala	Glu	Gln	Met	Ser	Thr	Leu	Pro	Ile	Asp	Ala	Pro	Ser	Pro
	850														
Leu	Glu	Asn	Leu	Glu	Gln	Lys	Glu	Thr	Pro	Gly	Ser	Gln	Pro	Ser	Glu
	865														880
Pro	Cys	Ser	Gly	Val	Ser	Arg	Gln	Glu	Glu	Ala	Lys	Ala	Ala	Val	Gly
															895
Val	Thr	Gly	Asn	Asp	Ile	Thr	Thr	Pro	Pro	Asn	Lys	Glu	Pro	Pro	Pro
Ser	Pro	Glu	Lys	Lys	Ala	Lys	Pro	Leu	Ala	Thr	Thr	Gln	Pro	Ala	Lys
Thr	Ser	Thr	Ser	Lys	Ala	Lys	Thr	Gln	Pro	Thr	Ser	Leu	Pro	Lys	Gln
Pro	Ala	Pro	Thr	Thr	Ser	Gly	Gly	Leu	Asn	Lys	Lys	Pro	Met	Ser	Leu
	945														960
Ala	Ser	Gly	Ser	Val	Pro	Ala	Ala	Pro	His	Lys	Arg	Pro	Ala	Ala	Ala
Thr	Ala	Thr	Ala	Arg	Pro	Ser	Thr	Leu	Pro	Ala	Arg	Asp	Val	Lys	Pro

Lys Pro Ile Thr Glu Ala Lys Val Ala Glu Lys Arg Thr Ser Pro Ser
 995 1000 1005
 Lys Pro Ser Ser Ala Pro Ala Leu Lys Pro Gly Pro Lys Thr Thr Pro
 1010 1015 1020
 Thr Val Ser Lys Ala Thr Ser Pro Ser Thr Leu Val Ser Thr Gly Pro
 1025 1030 1035 1040
 Ser Ser Arg Ser Pro Ala Thr Thr Leu Pro Lys Arg Pro Thr Ser Ile
 1045 1050 1055
 Lys Thr Glu Gly Lys Pro Ala Asp Val Lys Arg Met Thr Ala Lys Ser
 1060 1065 1070
 Ala Ser Ala Asp Leu Ser Arg Ser Lys Thr Thr Ser Ala Ser Ser Val
 1075 1080 1085
 Lys Arg Asn Thr Thr Pro Thr Gly Ala Ala Pro Pro Ala Gly Met Thr
 1090 1095 1100
 Ser Thr Arg Val Lys Pro Met Ser Ala Pro Ser Arg Ser Ser Gly Ala
 1105 1110 1115 1120
 Leu Ser Val Asp Lys Lys Pro Thr Ser Thr Lys Pro Ser Ser Ser Ala
 1125 1130 1135
 Pro Arg Val Ser Arg Leu Ala Thr Thr Val Ser Ala Pro Asp Leu Lys
 1140 1145 1150
 Ser Val Arg Ser Lys Val Gly Ser Thr Glu Asn Ile Lys His Gln Pro
 1155 1160 1165
 Gly Gly Gly Arg Ala Lys Val Glu Lys Lys Thr Glu Ala Ala Thr Thr
 1170 1175 1180
 Ala Gly Lys Pro Glu Pro Asn Ala Val Thr Lys Ala Ala Gly Ser Ile
 1185 1190 1195 1200
 Ala Ser Ala Gln Lys Pro Pro Ala Gly Lys Val Gln Ile Val Ser Lys
 1205 1210 1215
 Lys Val Ser Tyr Ser His Ile Gln Ser Lys Cys Val Ser Lys Asp Asn
 1220 1225 1230
 Ile Lys His Val Pro Gly Cys Gly Asn Val Gln Ile Gln Asn Lys Lys
 1235 1240 1245
 Val Asp Ile Ser Lys Val Ser Ser Lys Cys Gly Ser Lys Ala Asn Ile
 1250 1255 1260
 Lys His Lys Pro Gly Gly Gly Asp Val Lys Ile Glu Ser Gln Lys Leu
 1265 1270 1275 1280
 Asn Phe Lys Glu Lys Ala Gln Ala Lys Val Gly Ser Leu Asp Asn Val
 1285 1290 1295
 Gly His Phe Pro Ala Gly Gly Ala Val Lys Thr Glu Gly Gly Gly Ser
 1300 1305 1310

Glu Ala Leu Pro Cys Pro Gly Pro Pro Ala Gly Glu Glu Pro Val Ile
 1315 1320 1325
 Pro Glu Ala Ala Pro Asp Arg Gly Ala Pro Thr Ser Ala Ser Gly Leu
 1330 1335 1340
 Ser Gly His Thr Thr Leu Ser Gly Gly Gly Asp Gln Arg Glu Pro Gln
 1345 1350 1355 1360
 Thr Leu Asp Ser Gln Ile Gln Glu Thr Ser Ile Met Val Ser Lys Gly
 1365 1370 1375
 Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly
 1380 1385 1390
 Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp
 1395 1400 1405
 Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys
 1410 1415 1420
 Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu Thr His Gly Val
 1425 1430 1435 1440
 Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His Asp Phe Phe
 1445 1450 1455
 Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe
 1460 1465 1470
 Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly
 1475 1480 1485
 Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu
 1490 1495 1500
 Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Phe Asn Ser His
 1505 1510 1515 1520
 Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn
 1525 1530 1535
 Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp
 1540 1545 1550
 His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro
 1555 1560 1565
 Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn
 1570 1575 1580
 Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly
 1585 1590 1595 1600
 Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
 1605 1610

<210> 23
 <211> 978
 <212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(978)

<220>

<223> Description of Artificial Sequence:

GFP-nucleolus-Caspase 8-annexin II construct

<400> 23

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt	48
Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
ggt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc	528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	

195	200	205	
tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt			672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe			
210	215	220	
gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc			720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser			
225	230	235	240
gga aga aaa cgt ata cgt act tac ctc aag tcc tgc agg cgg atg aaa			768
Gly Arg Lys Arg Ile Arg Thr Tyr Leu Lys Ser Cys Arg Arg Met Lys			
	245	250	255
aga agt ggt ttt gag atg tct cga cct att cct tcc cac ctt act cga			816
Arg Ser Gly Phe Glu Met Ser Arg Pro Ile Pro Ser His Leu Thr Arg			
	260	265	270
tcg gca ggt gtt gaa aca gac gca ggt gtt gaa aca gac gca ggt gtt			864
Ser Ala Gly Val Glu Thr Asp Ala Gly Val Glu Thr Asp Ala Gly Val			
	275	280	285
gaa aca gac gca ggt gtt gaa aca gac gca ggt agt act atg tct act			912
Glu Thr Asp Ala Gly Val Glu Thr Asp Ala Gly Ser Thr Met Ser Thr			
	290	295	300
gtc cac gaa atc ctg tgc aag ctc agc ttg gag ggt gtt cat tct aca			960
Val His Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Val His Ser Thr			
305	310	315	320
ccc cca agt gcc gga tcc			978
Pro Pro Ser Ala Gly Ser			
	325		

<210> 24

<211> 326

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

GFP-nucleolus-Caspase 8-annexin II construct

<400> 24

Met	Ala	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu
1				5					10					15	

Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly
			20					25					30		

Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile
		35					40					45			

Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr
	50					55					60				

Leu	Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys
65					70					75				80	

Arg	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

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<210> 25
<211> 948
<212> DNA
<213> Artificial Sequence
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<220>  
<221> CDS  
<222> (1)..(948)
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<220>
<223> Description of Artificial Sequence:
GFP-nucleolus-Caspase 3-annexin II construct

<400> 25

atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt	48
Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu	
1 5 10 15	
ggt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga	96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly	
20 25 30	
gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc	144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile	
35 40 45	
tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa	240
Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa	288
Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc	528
Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195 200 205	
tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tcc	720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser	
225 230 235 240	

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gga aga aaa cgt ata cgt act tac ctc aag tcc tgc agg cgg atg aaa 768
Gly Arg Lys Arg Ile Arg Thr Tyr Leu Lys Ser Cys Arg Arg Met Lys
                245                      250                      255

aga agt ggt ttt gag atg tct cga cct att cct tcc cac ctt act cga 816
Arg Ser Gly Phe Glu Met Ser Arg Pro Ile Pro Ser His Leu Thr Arg
                260                      265                      270

tcg tat gaa aaa gga ata cca gtt gaa aca gac agc gaa gag caa gct 864
Ser Tyr Glu Lys Gly Ile Pro Val Glu Thr Asp Ser Glu Glu Gln Ala
                275                      280                      285

tat agt act atg tct act gtc cac gaa atc ctg tgc aag ctc agc ttg 912
Tyr Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu
                290                      295                      300

gag ggt gtt cat tct aca ccc cca agt gcc gga tcc 948
Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser
305                      310                      315

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<210> 26

<211> 316

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

GFP-nucleolus-Caspase 3-annexin II construct

<400> 26

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Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1              5              10              15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
                20              25              30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35              40              45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50              55              60

Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65              70              75              80

Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                85              90              95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
                100             105             110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
                115             120             125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
                130             135             140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145              150              155              160

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Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
 165 170 175
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220
 Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn Ser
 225 230 235 240
 Gly Arg Lys Arg Ile Arg Thr Tyr Leu Lys Ser Cys Arg Arg Met Lys
 245 250 255
 Arg Ser Gly Phe Glu Met Ser Arg Pro Ile Pro Ser His Leu Thr Arg
 260 265 270
 Ser Tyr Glu Lys Gly Ile Pro Val Glu Thr Asp Ser Glu Glu Gln Ala
 275 280 285
 Tyr Ser Thr Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu
 290 295 300
 Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser
 305 310 315

<210> 27

<211> 2088

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(1041)

<220>

<223> Description of Artificial Sequence:
 NLS-Fred25-synaptobrevin construct

<400> 27

atg aga aga aaa cga caa aag gct agc aaa gga gaa gaa ctc ttc act 48
 Met Arg Arg Lys Arg Gln Lys Ala Ser Lys Gly Glu Glu Leu Phe Thr
 1 5 10 15
 gga gtt gtc cca att ctt gtt gaa tta gat ggt gat gtt aac ggc cac 96
 Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His
 20 25 30
 aag ttc tct gtc agt gga gag ggt gaa ggt gat gca aca tac gga aaa 144
 Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys
 35 40 45
 ctt acc ctg aag ttc atc tgc act act ggc aaa ctg cct gtt cca tgg 192
 Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp
 50 55 60

cca	aca	cta	gtc	act	act	ctg	tgc	tat	ggg	gtt	caa	tgc	ttt	tca	aga	240
Pro	Thr	Leu	Val	Thr	Thr	Leu	Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	
65					70					75					80	
tac	ccg	gat	cat	atg	aaa	cgg	cat	gac	ttt	ttc	aag	agt	gcc	atg	ccc	288
Tyr	Pro	Asp	His	Met	Lys	Arg	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	
				85					90						95	
gaa	ggg	tat	gta	cag	gaa	agg	acc	atc	ttc	ttc	aaa	gat	gac	ggc	aac	336
Glu	Gly	Tyr	Val	Gln	Glu	Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	
			100					105					110			
tac	aag	aca	cgt	gct	gaa	gtc	aag	ttt	gaa	ggg	gat	acc	ctt	gtt	aat	384
Tyr	Lys	Thr	Arg	Ala	Glu	Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	
		115					120					125				
aga	atc	gag	tta	aaa	ggg	att	gac	ttc	aag	gaa	gat	ggc	aac	att	ctg	432
Arg	Ile	Glu	Leu	Lys	Gly	Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	
	130					135					140					
gga	cac	aaa	ttg	gaa	tac	aac	tat	aac	tca	cac	aat	gta	tac	atc	atg	480
Gly	His	Lys	Leu	Glu	Tyr	Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	
145					150					155					160	
gca	gac	aaa	caa	aag	aat	gga	atc	aaa	gtg	aac	ttc	aag	acc	cgc	cac	528
Ala	Asp	Lys	Gln	Lys	Asn	Gly	Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His	
				165					170					175		
aac	att	gaa	gat	gga	agc	gtt	caa	cta	gca	gac	cat	tat	caa	caa	aat	576
Asn	Ile	Glu	Asp	Gly	Ser	Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	
			180					185					190			
act	cca	att	ggc	gat	ggc	cct	gtc	ctt	tta	cca	gac	aac	cat	tac	ctg	624
Thr	Pro	Ile	Gly	Asp	Gly	Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	
		195					200					205				
tcc	aca	caa	tct	gcc	ctt	tgc	aaa	gat	ccc	aac	gaa	aag	aga	gac	cac	672
Ser	Thr	Gln	Ser	Ala	Leu	Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	
	210					215					220					
atg	gtc	ctt	ctt	gag	ttt	gta	aca	gct	gct	ggg	att	aca	cat	ggc	atg	720
Met	Val	Leu	Leu	Glu	Phe	Val	Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	
225					230					235					240	
gat	gaa	ctg	tac	aac	acc	ggg	atg	tct	aca	ggg	cca	act	gct	gcc	act	768
Asp	Glu	Leu	Tyr	Asn	Thr	Gly	Met	Ser	Thr	Gly	Pro	Thr	Ala	Ala	Thr	
				245					250					255		
ggc	agt	aat	cga	aga	ctt	cag	cag	aca	caa	aat	caa	gta	gat	gag	gtg	816
Gly	Ser	Asn	Arg	Arg	Leu	Gln	Gln	Thr	Gln	Asn	Gln	Val	Asp	Glu	Val	
			260					265					270			
gtg	gac	ata	atg	cga	gtt	aac	gtg	gac	aag	gtt	ctg	gaa	aga	gac	cag	864
Val	Asp	Ile	Met	Arg	Val	Asn	Val	Asp	Lys	Val	Leu	Glu	Arg	Asp	Gln	
		275					280					285				
aag	ctc	tct	gag	tta	gac	gac	cgt	gca	gac	gca	ctg	cag	gca	ggc	gct	912
Lys	Leu	Ser	Glu	Leu	Asp	Asp	Arg	Ala	Asp	Ala	Leu	Gln	Ala	Gly	Ala	
	290					295					300					
tct	caa	ttt	gaa	acg	agc	gca	gcc	aag	ttg	aag	agg	aaa	tat	tgg	tgg	960

Ser Gln Phe Glu Thr Ser Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp
 305 310 315 320
 aag aat tgc aag atg tgg gca atc ggg att act gtt ctg gtt atc ttc 1008
 Lys Asn Cys Lys Met Trp Ala Ile Gly Ile Thr Val Leu Val Ile Phe
 325 330 335
 atc atc atc atc atc gtg tgg gtt gtc tct tca tgaatgagaa gaaaacgaca 1061
 Ile Ile Ile Ile Ile Val Trp Val Val Ser Ser
 340 345
 aaaggctagc aaaggagaag aactcttcac tggagttgtc ccaattcttg ttgaattaga 1121
 tgggtgatgtt aacggccaca agttctctgt cagtggagag ggtgaagggtg atgcaacata 1181
 cggaataactt accctgaagt tcatctgcac tactggcaaa ctgcctgttc catggccaac 1241
 actagtcact actctgtgct atgggtgttca atgcttttca agatacccg atcatatgaa 1301
 acggcatgac tttttcaaga gtgccatgcc cgaagggttat gtacaggaaa ggaccatctt 1361
 cttcaaagat gacggcaact acaagacacg tgctgaagtc aagtttgaag gtgataccct 1421
 tgtaataaga atcgagttaa aaggatttga cttcaaggaa gatggcaaca ttctgggaca 1481
 caaattggaa tacaactata actcacacaa tgtatacatc atggcagaca aaaaaagaa 1541
 tggaatcaaa gtgaacttca agacccgcc caacattgaa gatggaagcg ttcaactagc 1601
 agaccattat caacaaaata ctccaattgg cgatggccct gtccttttac cagacaacca 1661
 ttacctgtcc acacaatctg ccctttcgaa agatcccaac gaaaagagag accacatggg 1721
 ccttcttgag tttgtaacag ctgctgggat tacacatggc atggatgaac tgtacaacac 1781
 cggatgtct acaggtccaa ctgctgccac tggcagtaat cgaagacttc agcagacaca 1841
 aaatcaagta gatgaggtgg tggacataat gcgagttaac gtggacaagg ttctggaaag 1901
 agaccagaag ctctctgagt tagacgaccg tgcagacgca ctgcaggcag gcgcttctca 1961
 atttgaaacg agcgagcca agttgaagag gaaatattgg tggaagaatt gcaagatgtg 2021
 ggcaatcggg attactgttc tggttatctt catcatcatc atcatcgtgt gggttgtctc 2081
 ttcatga 2088

<210> 28

<211> 347

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
 NLS-Fred25-synaptobrevin construct

<400> 28

Met Arg Arg Lys Arg Gln Lys Ala Ser Lys Gly Glu Glu Leu Phe Thr
 1 5 10 15

Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His
 20 25 30
 Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys
 35 40 45
 Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp
 50 55 60
 Pro Thr Leu Val Thr Thr Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg
 65 70 75 80
 Tyr Pro Asp His Met Lys Arg His Asp Phe Phe Lys Ser Ala Met Pro
 85 90 95
 Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn
 100 105 110
 Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn
 115 120 125
 Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu
 130 135 140
 Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met
 145 150 155 160
 Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Thr Arg His
 165 170 175
 Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn
 180 185 190
 Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu
 195 200 205
 Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His
 210 215 220
 Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met
 225 230 235 240
 Asp Glu Leu Tyr Asn Thr Gly Met Ser Thr Gly Pro Thr Ala Ala Thr
 245 250 255
 Gly Ser Asn Arg Arg Leu Gln Gln Thr Gln Asn Gln Val Asp Glu Val
 260 265 270
 Val Asp Ile Met Arg Val Asn Val Asp Lys Val Leu Glu Arg Asp Gln
 275 280 285
 Lys Leu Ser Glu Leu Asp Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala
 290 295 300
 Ser Gln Phe Glu Thr Ser Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp
 305 310 315 320
 Lys Asn Cys Lys Met Trp Ala Ile Gly Ile Thr Val Leu Val Ile Phe
 325 330 335
 Ile Ile Ile Ile Ile Val Trp Val Val Ser Ser

340

345

<210> 29
 <211> 2106
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1) .. (1050)

<220>
 <223> Description of Artificial Sequence:
 NLS-Fred25-cellubrevin construct

<400> 29
 atg aga aga aaa cga caa aag gct agc aaa gga gaa gaa ctc ttc act 48
 Met Arg Arg Lys Arg Gln Lys Ala Ser Lys Gly Glu Glu Leu Phe Thr
 1 5 10 15
 gga gtt gtc cca att ctt gtt gaa tta gat ggt gat gtt aac ggc cac 96
 Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His
 20 25 30
 aag ttc tct gtc agt gga gag ggt gaa ggt gat gca aca tac gga aaa 144
 Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys
 35 40 45
 ctt acc ctg aag ttc atc tgc act act ggc aaa ctg cct gtt cca tgg 192
 Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp
 50 55 60
 cca aca cta gtc act act ctg tgc tat ggt gtt caa tgc ttt tca aga 240
 Pro Thr Leu Val Thr Thr Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg
 65 70 75 80
 tac ccg gat cat atg aaa cgg cat gac ttt ttc aag agt gcc atg ccc 288
 Tyr Pro Asp His Met Lys Arg His Asp Phe Phe Lys Ser Ala Met Pro
 85 90 95
 gaa ggt tat gta cag gaa agg acc atc ttc ttc aaa gat gac ggc aac 336
 Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn
 100 105 110
 tac aag aca cgt gct gaa gtc aag ttt gaa ggt gat acc ctt gtt aat 384
 Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn
 115 120 125
 aga atc gag tta aaa ggt att gac ttc aag gaa gat ggc aac att ctg 432
 Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu
 130 135 140
 gga cac aaa ttg gaa tac aac tat aac tca cac aat gta tac atc atg 480
 Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met
 145 150 155 160
 gca gac aaa caa aag aat gga atc aaa gtg aac ttc aag acc cgc cac 528
 Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Thr Arg His
 165 170 175

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aac att gaa gat gga agc gtt caa cta gca gac cat tat caa caa aat 576
Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn
180 185 190

act cca att ggc gat ggc cct gtc ctt tta cca gac aac cat tac ctg 624
Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu
195 200 205

tcc aca caa tct gcc ctt tcg aaa gat ccc aac gaa aag aga gac cac 672
Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His
210 215 220

atg gtc ctt ctt gag ttt gta aca gct gct ggg att aca cat ggc atg 720
Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr His Gly Met
225 230 235 240

gat gaa ctg tac aac acc ggt atg tct aca ggt gtg cct tcg ggg tca 768
Asp Glu Leu Tyr Asn Thr Gly Met Ser Thr Gly Val Pro Ser Gly Ser
245 250 255

agt gct gcc act ggc agt aat cga aga ctc cag cag aca caa aat caa 816
Ser Ala Ala Thr Gly Ser Asn Arg Arg Leu Gln Gln Thr Gln Asn Gln
260 265 270

gta gat gag gtg gtt gac atc atg aga gtc aat gtg gat aag gtg tta 864
Val Asp Glu Val Val Asp Ile Met Arg Val Asn Val Asp Lys Val Leu
275 280 285

gaa aga gac cag aag ctc tcg gag cta gat gac cgc gca gat gca ctg 912
Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp Asp Arg Ala Asp Ala Leu
290 295 300

cag gca ggt gcc tcg cag ttt gaa aca agt gct gcc aag ttg aag aga 960
Gln Ala Gly Ala Ser Gln Phe Glu Thr Ser Ala Ala Lys Leu Lys Arg
305 310 315 320

aag tat tgg tgg aag aac tgc aag atg tgg gcg ata ggg atc agt gtc 1008
Lys Tyr Trp Trp Lys Asn Cys Lys Met Trp Ala Ile Gly Ile Ser Val
325 330 335

ctg gtg atc att gtc atc atc atc atc atc gtg tgg tgt gtc tct 1050
Leu Val Ile Ile Val Ile Ile Ile Ile Val Trp Cys Val Ser
340 345 350

taa atg agaa gaaa acgaca aaaggctagc aaaggagaag aactcttcac tggagttgtc 1110

ccaattcttg ttgaattaga tggatgatgtt aacggccaca agttctctgt cagtggagag 1170

ggtgaagggtg atgcaacata cggaaaactt accctgaagt tcatctgcac tactggcaaa 1230

ctgcctgttc catggccaac actagtcact actctgtgct atgggtgttca atgctttttca 1290

agatacccggt atcatatgaa acggcatgac tttttcaaga gtgccatgcc cgaagggttat 1350

gtacaggaaa ggaccatctt cttcaaagat gacggcaact acaagacacg tgctgaagtc 1410

aagtttgaag gtgataccct tggttaataga atcgagttaa aaggatttga cttcaaggaa 1470

gatggcaaca ttctgggaca caaattggaa tacaactata actcacacaa tgtatacatc 1530

atggcagaca aacaaaagaa tggaatcaaa gtgaacttca agaccgcga caacattgaa 1590

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gatggaagcg ttcaactagc agaccattat caacaaaata ctccaattgg cgatggccct 1650
 gtcccttttac cagacaacca ttacctgtcc acacaatctg ccctttcgaa agatcccaac 1710
 gaaaagagag accacatggg ccttcttgag tttgtaacag ctgctgggat tacacatggc 1770
 atggatgaac tgtacaacac cggtatgtct acaggtgtgc cttcgggggc aagtgtgtgc 1830
 actggcagta atcgaagact ccagcagaca caaaatcaag tagatgaggt ggttgacatc 1890
 atgagagtca atgtggataa ggtgtagaa agagaccaga agctctcgga gctagatgac 1950
 cgcgcagatg cactgcaggc aggtgcctcg cagtttgaaa caagtgtgc caagttgaag 2010
 agaaagtatt ggtggaagaa ctgcaagatg tgggcgatag ggatcagtgt cctggtgatc 2070
 attgtcatca tcatcatcgt gtggtgtgtc tottaa 2106

<210> 30

<211> 350

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
NLS-Fred25-cellubrevin construct

<400> 30

Met	Arg	Arg	Lys	Arg	Gln	Lys	Ala	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr
1				5					10					15	

Gly	Val	Val	Pro	Ile	Leu	Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His
			20					25					30		

Lys	Phe	Ser	Val	Ser	Gly	Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys
		35					40					45			

Leu	Thr	Leu	Lys	Phe	Ile	Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp
	50					55					60				

Pro	Thr	Leu	Val	Thr	Thr	Leu	Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg
65					70					75					80

Tyr	Pro	Asp	His	Met	Lys	Arg	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro
				85					90					95	

Glu	Gly	Tyr	Val	Gln	Glu	Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn
			100					105					110		

Tyr	Lys	Thr	Arg	Ala	Glu	Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn
		115					120					125			

Arg	Ile	Glu	Leu	Lys	Gly	Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu
	130					135						140			

Gly	His	Lys	Leu	Glu	Tyr	Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met
145					150					155					160

Ala	Asp	Lys	Gln	Lys	Asn	Gly	Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

165										170					175				
Asn	Ile	Glu	Asp	Gly	Ser	Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn				
			180						185				190						
Thr	Pro	Ile	Gly	Asp	Gly	Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu				
		195					200					205							
Ser	Thr	Gln	Ser	Ala	Leu	Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His				
	210					215					220								
Met	Val	Leu	Leu	Glu	Phe	Val	Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met				
225					230					235					240				
Asp	Glu	Leu	Tyr	Asn	Thr	Gly	Met	Ser	Thr	Gly	Val	Pro	Ser	Gly	Ser				
				245					250					255					
Ser	Ala	Ala	Thr	Gly	Ser	Asn	Arg	Arg	Leu	Gln	Gln	Thr	Gln	Asn	Gln				
			260					265					270						
Val	Asp	Glu	Val	Val	Asp	Ile	Met	Arg	Val	Asn	Val	Asp	Lys	Val	Leu				
		275					280					285							
Glu	Arg	Asp	Gln	Lys	Leu	Ser	Glu	Leu	Asp	Asp	Arg	Ala	Asp	Ala	Leu				
	290					295					300								
Gln	Ala	Gly	Ala	Ser	Gln	Phe	Glu	Thr	Ser	Ala	Ala	Lys	Leu	Lys	Arg				
305					310					315					320				
Lys	Tyr	Trp	Trp	Lys	Asn	Cys	Lys	Met	Trp	Ala	Ile	Gly	Ile	Ser	Val				
				325					330					335					
Leu	Val	Ile	Ile	Val	Ile	Ile	Ile	Val	Trp	Cys	Val	Ser							
		340					345					350							

<210> 31

<211> 3171

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(3168)

<220>

<223> Description of Artificial Sequence:

NLS-EYFP-MAPKDM-EBFP construct

<400> 31

atg	agg	ccc	aga	aga	aag	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	48
Met	Arg	Pro	Arg	Arg	Lys	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	
1				5					10				15			

gtg	gtg	ccc	atc	ctg	gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	96
Val	Val	Pro	Ile	Leu	Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	
			20					25					30			

ttc	agc	gtg	tcc	ggc	gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	144
Phe	Ser	Val	Ser	Gly	Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	
		35					40					45				

acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc	192
Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro	
50 55 60	
acc ctc gtg acc acc ttc ggc tac ggc ctg cag tgc ttc gcc cgc tac	240
Thr Leu Val Thr Thr Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr	
65 70 75 80	
ccc gac cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa	288
Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu	
85 90 95	
ggc tac gtc cag gag cgc acc atc ttc ttc aag gac gac ggc aac tac	336
Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr	
100 105 110	
aag acc cgc gcc gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc	384
Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg	
115 120 125	
atc gag ctg aag ggc atc gac ttc aag gag gac ggc aac atc ctg ggg	432
Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly	
130 135 140	
cac aag ctg gag tac aac tac aac agc cac aac gtc tat atc atg gcc	480
His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala	
145 150 155 160	
gac aag cag aag aac ggc atc aag gtg aac ttc aag atc cgc cac aac	528
Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn	
165 170 175	
atc gag gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc	576
Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr	
180 185 190	
ccc atc ggc gac ggc ccc gtg ctg ctg ccg gac aac cac tac ctg agc	624
Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser	
195 200 205	
tac cag tcc gcc ctg agc aaa gac ccc aac gag aag cgc gat cac atg	672
Tyr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met	
210 215 220	
gtc ctg ctg gag ttc gtg acc gcc gcc ggg atc act ctc ggc atg gac	720
Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp	
225 230 235 240	
gag ctg tac aag aag gga gac gaa gtg gac gga gcc gac ctc agt ctt	768
Glu Leu Tyr Lys Lys Gly Asp Glu Val Asp Gly Ala Asp Leu Ser Leu	
245 250 255	
gtg gat gcg ttg aca gaa cca cct cca gaa att gag gga gaa ata aag	816
Val Asp Ala Leu Thr Glu Pro Pro Pro Glu Ile Glu Gly Glu Ile Lys	
260 265 270	
cga gac ttc atg gct gcg ctg gag gca gag ccc tat gat gac atc gtg	864
Arg Asp Phe Met Ala Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val	
275 280 285	

gga gaa act gtg gag aaa act gag ttt att cct ctc ctg gat ggt gat	912
Gly Glu Thr Val Glu Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp	
290 295 300	
gag aaa acc ggg aac tca gag tcc aaa aag aaa ccc tgc tta gac act	960
Glu Lys Thr Gly Asn Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr	
305 310 315 320	
agc cag gtt gaa ggt atc cca tct tct aaa cca aca ctc cta gcc aat	1008
Ser Gln Val Glu Gly Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn	
325 330 335	
ggt gat cat gga atg gag ggg aat aac act gca ggg tct cca act gac	1056
Gly Asp His Gly Met Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp	
340 345 350	
ttc ctt gaa gag aga gtg gac tat ccg gat tat cag agc agc cag aac	1104
Phe Leu Glu Glu Arg Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn	
355 360 365	
tgg cca gaa gat gca agc ttt tgt ttc cag cct cag caa gtg tta gat	1152
Trp Pro Glu Asp Ala Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp	
370 375 380	
act gac cag gct gag ccc ttt aac gag cac cgt gat gat ggt ttg gca	1200
Thr Asp Gln Ala Glu Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala	
385 390 395 400	
gat ctg ctc ttt gtc tcc agt gga ccc acg aac gct tct gca ttt aca	1248
Asp Leu Leu Phe Val Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr	
405 410 415	
gag cga gac aat cct tca gaa gac agt tac ggt atg ctt ccc tgt gac	1296
Glu Arg Asp Asn Pro Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp	
420 425 430	
tca ttt gct tcc acg gct gtt gta tct cag gag tgg tct gtg gga gcc	1344
Ser Phe Ala Ser Thr Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala	
435 440 445	
cca aac tct cca tgt tca gag tcc tgt gtc tcc cca gag gtt act ata	1392
Pro Asn Ser Pro Cys Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile	
450 455 460	
gaa acc cta cag cca gca aca gag ctc tcc aag gca gca gaa gtg gaa	1440
Glu Thr Leu Gln Pro Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu	
465 470 475 480	
tca gtg aaa gag cag ctg cca gct aaa gca ttg gaa acg atg gca gag	1488
Ser Val Lys Glu Gln Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu	
485 490 495	
cag acc act gat gtg gtg cac tct cca tcc aca gac aca aca cca ggc	1536
Gln Thr Thr Asp Val Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly	
500 505 510	
cca gac aca gag gca gca ctg gct aaa gac ata gaa gag atc acc aag	1584
Pro Asp Thr Glu Ala Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys	
515 520 525	
cca gat gtg ata ttg gca aat gtc acg cag cca tct act gaa tcg gat	1632

Pro	Asp	Val	Ile	Leu	Ala	Asn	Val	Thr	Gln	Pro	Ser	Thr	Glu	Ser	Asp		
530						535					540						
atg	ttc	ctg	gcc	cag	gac	atg	gaa	cta	ctc	aca	gga	aca	gag	gca	gcc	1680	
Met	Phe	Leu	Ala	Gln	Asp	Met	Glu	Leu	Leu	Thr	Gly	Thr	Glu	Ala	Ala		
545					550					555					560		
cac	gct	aac	aat	atc	ata	ttg	cct	aca	gaa	cca	gac	gaa	tct	tca	acc	1728	
His	Ala	Asn	Asn	Ile	Ile	Leu	Pro	Thr	Glu	Pro	Asp	Glu	Ser	Ser	Thr		
				565					570					575			
aag	gat	gta	gca	cca	cct	atg	gaa	gaa	gaa	att	gtc	cca	ggc	aat	gat	1776	
Lys	Asp	Val	Ala	Pro	Pro	Met	Glu	Glu	Glu	Ile	Val	Pro	Gly	Asn	Asp		
			580					585					590				
acg	aca	tcc	ccc	aaa	gaa	aca	gag	aca	aca	ctt	cca	ata	aaa	atg	gac	1824	
Thr	Thr		Pro	Lys	Glu	Thr	Glu	Thr	Thr	Leu	Pro	Ile	Lys	Met	Asp		
		595					600					605					
ttg	gca	cca	cct	gag	gat	gtg	tta	ctt	acc	aaa	gaa	aca	gaa	cta	gcc	1872	
Leu	Ala	Pro	Pro	Glu	Asp	Val	Leu	Leu	Thr	Lys	Glu	Thr	Glu	Leu	Ala		
	610					615					620						
cca	gcc	aag	ggc	atg	gtt	tca	ctc	tca	gaa	ata	gaa	gag	gct	ctg	gca	1920	
Pro	Ala	Lys	Gly	Met	Val	Ser	Leu	Ser	Glu	Ile	Glu	Glu	Ala	Leu	Ala		
625					630					635					640		
aag	aat	gat	gtt	cgc	tct	gca	gaa	ata	cct	gtg	gct	cag	gag	aca	gtg	1968	
Lys	Asn	Asp	Val	Arg	Ser	Ala	Glu	Ile	Pro	Val	Ala	Gln	Glu	Thr	Val		
				645					650					655			
gtc	tca	gaa	aca	gag	gtg	gtc	ctg	gca	aca	gaa	gtg	gta	ctg	ccc	tca	2016	
Val	Ser	Glu	Thr	Glu	Val	Val	Leu	Ala	Thr	Glu	Val	Val	Leu	Pro	Ser		
			660					665					670				
gat	ccc	ata	aca	aca	ttg	aca	aag	gat	gtg	aca	ctc	ccc	tta	gaa	gca	2064	
Asp	Pro	Ile	Thr	Thr	Leu	Thr	Lys	Asp	Val	Thr	Leu	Pro	Leu	Glu	Ala		
		675					680					685					
gag	aga	ccg	ttg	gtg	acg	gac	atg	act	cca	tct	ctg	gaa	aca	gaa	atg	2112	
Glu	Arg	Pro	Leu	Val	Thr	Asp	Met	Thr	Pro	Ser	Leu	Glu	Thr	Glu	Met		
	690					695					700						
acc	cta	ggc	aaa	gag	aca	gct	cca	ccc	aca	gaa	aca	aat	ttg	ggc	atg	2160	
Thr	Leu	Gly	Lys	Glu	Thr	Ala	Pro	Pro	Thr	Glu	Thr	Asn	Leu	Gly	Met		
	705				710					715					720		
gcc	aaa	gac	atg	tct	cca	ctc	cca	gaa	tca	gaa	gtg	act	ctg	ggc	aag	2208	
Ala	Lys	Asp	Met	Ser	Pro	Leu	Pro	Glu	Ser	Glu	Val	Thr	Leu	Gly	Lys		
				725					730					735			
gac	gtg	gtt	ata	ctt	cca	gaa	aca	aag	gtg	gct	gag	ttt	aac	aat	gtg	2256	
Asp	Val	Val	Ile	Leu	Pro	Glu	Thr	Lys	Val	Ala	Glu	Phe	Asn	Asn	Val		
			740					745					750				
act	cca	ctt	tca	gaa	gaa	gag	gta	acc	tca	gtc	aag	gac	atg	tct	ccg	2304	
Thr	Pro	Leu	Ser	Glu	Glu	Glu	Val	Thr	Ser	Val	Lys	Asp	Met	Ser	Pro		
		755					760					765					
tct	gca	gaa	aca	gag	gct	ccc	ctg	gct	aag	aat	gct	gat	ctg	cac	tca	2352	
Ser	Ala	Glu	Thr	Glu	Ala	Pro	Leu	Ala	Lys	Asn	Ala	Asp	Leu	His	Ser		

770	775	780	
gga aca gag ctg att gtg gac aac agc atg gct cca gcc tcc gat ctt Gly Thr Glu Leu Ile Val Asp Asn Ser Met Ala Pro Ala Ser Asp Leu 785 790 795 800			2400
gca ctg ccc ttg gaa aca aaa gta gca aca gtt cca att aaa gac aaa Ala Leu Pro Leu Glu Thr Lys Val Ala Thr Val Pro Ile Lys Asp Lys 805 810 815			2448
gga atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc Gly Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile 820 825 830			2496
ctg gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser 835 840 845			2544
ggc gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe 850 855 860			2592
atc tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr 865 870 875 880			2640
acc ctg acc cac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg Thr Leu Thr His Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met 885 890 895			2688
aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln 900 905 910			2736
gag cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala 915 920 925			2784
gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys 930 935 940			2832
ggc atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu 945 950 955 960			2880
tac aac ttc aac agc cac aac gtc tat atc atg gcc gac aag cag aag Tyr Asn Phe Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys 965 970 975			2928
aac ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly 980 985 990			2976
agc gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp 995 1000 1005			3024
ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala 1010 1015 1020			3072

ctg agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag 3120
 Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu
 1025 1030 1035 1040

ttc gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag 3168
 Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
 1045 1050 1055

tag 3171

<210> 32

<211> 1056

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
 NLS-EYFP-MAPKDM-EBFP construct

<400> 32

Met Arg Pro Arg Arg Lys Val Ser Lys Gly Glu Glu Leu Phe Thr Gly
 1 5 10 15

Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys
 20 25 30

Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu
 35 40 45

Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro
 50 55 60

Thr Leu Val Thr Thr Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr
 65 70 75 80

Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu
 85 90 95

Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr
 100 105 110

Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg
 115 120 125

Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly
 130 135 140

His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala
 145 150 155 160

Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn
 165 170 175

Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr
 180 185 190

Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser
 195 200 205

Tyr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met
 210 215 220
 Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp
 225 230 235 240
 Glu Leu Tyr Lys Lys Gly Asp Glu Val Asp Gly Ala Asp Leu Ser Leu
 245 250 255
 Val Asp Ala Leu Thr Glu Pro Pro Glu Ile Glu Gly Glu Ile Lys
 260 265 270
 Arg Asp Phe Met Ala Ala Leu Glu Ala Glu Pro Tyr Asp Asp Ile Val
 275 280 285
 Gly Glu Thr Val Glu Lys Thr Glu Phe Ile Pro Leu Leu Asp Gly Asp
 290 295 300
 Glu Lys Thr Gly Asn Ser Glu Ser Lys Lys Lys Pro Cys Leu Asp Thr
 305 310 315 320
 Ser Gln Val Glu Gly Ile Pro Ser Ser Lys Pro Thr Leu Leu Ala Asn
 325 330 335
 Gly Asp His Gly Met Glu Gly Asn Asn Thr Ala Gly Ser Pro Thr Asp
 340 345 350
 Phe Leu Glu Glu Arg Val Asp Tyr Pro Asp Tyr Gln Ser Ser Gln Asn
 355 360 365
 Trp Pro Glu Asp Ala Ser Phe Cys Phe Gln Pro Gln Gln Val Leu Asp
 370 375 380
 Thr Asp Gln Ala Glu Pro Phe Asn Glu His Arg Asp Asp Gly Leu Ala
 385 390 395 400
 Asp Leu Leu Phe Val Ser Ser Gly Pro Thr Asn Ala Ser Ala Phe Thr
 405 410 415
 Glu Arg Asp Asn Pro Ser Glu Asp Ser Tyr Gly Met Leu Pro Cys Asp
 420 425 430
 Ser Phe Ala Ser Thr Ala Val Val Ser Gln Glu Trp Ser Val Gly Ala
 435 440 445
 Pro Asn Ser Pro Cys Ser Glu Ser Cys Val Ser Pro Glu Val Thr Ile
 450 455 460
 Glu Thr Leu Gln Pro Ala Thr Glu Leu Ser Lys Ala Ala Glu Val Glu
 465 470 475 480
 Ser Val Lys Glu Gln Leu Pro Ala Lys Ala Leu Glu Thr Met Ala Glu
 485 490 495
 Gln Thr Thr Asp Val Val His Ser Pro Ser Thr Asp Thr Thr Pro Gly
 500 505 510
 Pro Asp Thr Glu Ala Ala Leu Ala Lys Asp Ile Glu Glu Ile Thr Lys
 515 520 525
 Pro Asp Val Ile Leu Ala Asn Val Thr Gln Pro Ser Thr Glu Ser Asp

530					535					540					
Met	Phe	Leu	Ala	Gln	Asp	Met	Glu	Leu	Leu	Thr	Gly	Thr	Glu	Ala	Ala
545					550					555					560
His	Ala	Asn	Asn	Ile	Ile	Leu	Pro	Thr	Glu	Pro	Asp	Glu	Ser	Ser	Thr
				565					570					575	
Lys	Asp	Val	Ala	Pro	Pro	Met	Glu	Glu	Glu	Ile	Val	Pro	Gly	Asn	Asp
			580						585				590		
Thr	Thr	Ser	Pro	Lys	Glu	Thr	Glu	Thr	Thr	Leu	Pro	Ile	Lys	Met	Asp
		595					600					605			
Leu	Ala	Pro	Pro	Glu	Asp	Val	Leu	Leu	Thr	Lys	Glu	Thr	Glu	Leu	Ala
	610					615					620				
Pro	Ala	Lys	Gly	Met	Val	Ser	Leu	Ser	Glu	Ile	Glu	Glu	Ala	Leu	Ala
625					630					635					640
Lys	Asn	Asp	Val	Arg	Ser	Ala	Glu	Ile	Pro	Val	Ala	Gln	Glu	Thr	Val
				645					650					655	
Val	Ser	Glu	Thr	Glu	Val	Val	Leu	Ala	Thr	Glu	Val	Val	Leu	Pro	Ser
			660					665					670		
Asp	Pro	Ile	Thr	Thr	Leu	Thr	Lys	Asp	Val	Thr	Leu	Pro	Leu	Glu	Ala
		675					680					685			
Glu	Arg	Pro	Leu	Val	Thr	Asp	Met	Thr	Pro	Ser	Leu	Glu	Thr	Glu	Met
	690					695					700				
Thr	Leu	Gly	Lys	Glu	Thr	Ala	Pro	Pro	Thr	Glu	Thr	Asn	Leu	Gly	Met
705					710					715					720
Ala	Lys	Asp	Met	Ser	Pro	Leu	Pro	Glu	Ser	Glu	Val	Thr	Leu	Gly	Lys
				725					730					735	
Asp	Val	Val	Ile	Leu	Pro	Glu	Thr	Lys	Val	Ala	Glu	Phe	Asn	Asn	Val
			740					745					750		
Thr	Pro	Leu	Ser	Glu	Glu	Glu	Val	Thr	Ser	Val	Lys	Asp	Met	Ser	Pro
		755					760					765			
Ser	Ala	Glu	Thr	Glu	Ala	Pro	Leu	Ala	Lys	Asn	Ala	Asp	Leu	His	Ser
	770					775					780				
Gly	Thr	Glu	Leu	Ile	Val	Asp	Asn	Ser	Met	Ala	Pro	Ala	Ser	Asp	Leu
785					790					795					800
Ala	Leu	Pro	Leu	Glu	Thr	Lys	Val	Ala	Thr	Val	Pro	Ile	Lys	Asp	Lys
				805					810					815	
Gly	Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile
			820					825					830		
Leu	Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser
		835					840					845			
Gly	Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe
	850					855					860				

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Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr
865                               870                               875                               880

Thr Leu Thr His Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met
885                               890

Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln
900                               905                               910

Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala
915                               920                               925

Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys
930                               935                               940

Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu
945                               950                               955                               960

Tyr Asn Phe Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys
965                               970                               975

Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly
980                               985                               990

Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp
995                               1000                               1005

Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala
1010                               1015                               1020

Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu
1025                               1030                               1035                               1040

Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
1045                               1050                               1055

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<210> 33
 <211> 1623
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(1623)

<220>
 <223> Description of Artificial Sequence:
 YFP-NLS-CP3-multiple DEVD-CFP-Annexin II construct

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<400> 33
atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg   48
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
  1                5                10                15

gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc   96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
      20                25                30

gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc   144

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Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile		
		35					40					45					
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192	
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr		
	50					55				60							
ttc	ggc	tac	ggc	ctg	cag	tgc	ttc	gcc	cgc	tac	ccc	gac	cac	atg	aag	240	
Phe	Gly	Tyr	Gly	Leu	Gln	Cys	Phe	Ala	Arg	Tyr	Pro	Asp	His	Met	Lys		
65					70				75					80			
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288	
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu		
			85						90					95			
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu		
		100						105					110				
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly		
	115						120					125					
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr		
	130					135					140						
aac	tac	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn		
145					150					155					160		
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528	
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser		
			165					170						175			
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly		
			180					185					190				
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	tac	cag	tcc	gcc	ctg	624	
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Tyr	Gln	Ser	Ala	Leu		
		195					200					205					
agc	aaa	gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	672	
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe		
	210					215					220						
gtg	acc	gcc	gcc	ggg	atc	act	ctc	ggc	atg	gac	gag	ctg	tac	aag	tcc	720	
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Ser		
	225				230				235					240			
gga	aga	agg	aaa	cga	caa	aag	cga	tcg	gca	ggt	gac	gaa	gtt	gat	gca	768	
Gly	Arg	Arg	Lys	Arg	Gln	Lys	Arg	Ser	Ala	Gly	Asp	Glu	Val	Asp	Ala		
			245					250						255			
ggt	gac	gaa	gtt	gat	gca	ggt	gac	gaa	gtt	gat	gca	ggt	gac	gaa	gtt	816	
Gly	Asp	Glu	Val	Asp	Ala	Gly	Asp	Glu	Val	Asp	Ala	Gly	Asp	Glu	Val		
		260					265					270					
gac	gca	ggt	agt	act	atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	864	
Asp	Ala	Gly	Ser	Thr	Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly		

275	280	285	
gtg gtg ccc atc ctg gtc gag ctg gac ggc gac gta aac ggc cac aag Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys 290 295 300			912
ttc agc gtg tcc ggc gag ggc gag ggc gat gcc acc tac ggc aag ctg Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu 305 310 315 320			960
acc ctg aag ttc atc tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro 325 330 335			1008
acc ctg gtg acc acc ctg acc tgg ggc gtg cag tgc ttc agc cgc tac Thr Leu Val Thr Thr Leu Thr Trp Gly Val Gln Cys Phe Ser Arg Tyr 340 345 350			1056
ccc gac cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu 355 360 365			1104
ggc tac gtc cag gag cgc acc atc ttc ttc aag gac gac ggc aac tac Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr 370 375 380			1152
aag acc cgc gcc gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg 385 390 395 400			1200
atc gag ctg aag ggc atc gac ttc aag gag gac ggc aac atc ctg ggg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly 405 410 415			1248
cac aag ctg gag tac aac tac atc agc cac aac gtc tat atc acc gcc His Lys Leu Glu Tyr Asn Tyr Ile Ser His Asn Val Tyr Ile Thr Ala 420 425 430			1296
gac aag cag aag aac ggc atc aag gcc aac ttc aag atc cgc cac aac Asp Lys Gln Lys Asn Gly Ile Lys Ala Asn Phe Lys Ile Arg His Asn 435 440 445			1344
atc gag gac ggc agc gtg cag ctg gcc gac cac tac cag cag aac acc Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr 450 455 460			1392
ccc atc ggc gac ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser 465 470 475 480			1440
acc cag tcc gcc ctg agc aaa gac ccc aac gag aag cgc gat cac atg Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met 485 490 495			1488
gtc ctg ctg gag ttc gtg acc gcc gcc ggg atc act ctg ggc atg gac Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp 500 505 510			1536
gag ctg tac aag atg tct act gtc cac gaa atc ctg tgc aag ctg agc Glu Leu Tyr Lys Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser 515 520 525			1584

ttg gag ggt gtt cat tct aca ccc cca agt gcc gga tcc
 Leu Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser
 530 535 540

<210> 34

<211> 541

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

YFP-NLS-CP3-multiple DEVD-CFP-Annexin II construct

<400> 34

Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60

Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys
 65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
 165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu
 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
 225 230 235 240

Gly Arg Arg Lys Arg Gln Lys Arg Ser Ala Gly Asp Glu Val Asp Ala
 245 250 255
 Gly Asp Glu Val Asp Ala Gly Asp Glu Val Asp Ala Gly Asp Glu Val
 260 265 270
 Asp Ala Gly Ser Thr Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly
 275 280 285
 Val Val Pro Ile Leu Val Glu Leu Asp Gly Asp Val Asn Gly His Lys
 290 295 300
 Phe Ser Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu
 305 310 315 320
 Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro
 325 330 335
 Thr Leu Val Thr Thr Leu Thr Trp Gly Val Gln Cys Phe Ser Arg Tyr
 340 345 350
 Pro Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu
 355 360 365
 Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr
 370 375 380
 Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg
 385 390 395 400
 Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly
 405 410 415
 His Lys Leu Glu Tyr Asn Tyr Ile Ser His Asn Val Tyr Ile Thr Ala
 420 425 430
 Asp Lys Gln Lys Asn Gly Ile Lys Ala Asn Phe Lys Ile Arg His Asn
 435 440 445
 Ile Glu Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr
 450 455 460
 Pro Ile Gly Asp Gly Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser
 465 470 475 480
 Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met
 485 490 495
 Val Leu Leu Glu Phe Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp
 500 505 510
 Glu Leu Tyr Lys Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser
 515 520 525
 Leu Glu Gly Val His Ser Thr Pro Pro Ser Ala Gly Ser
 530 535 540

<210> 35
 <211> 24
 <212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: FLAG epitope

<400> 35

gactacaaag acgacgacga caaa

24

<210> 36

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: FLAG epitope

<400> 36

Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

<210> 37

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HA epitope

<400> 37

taccatagc acgtaccaga ctacgca

27

<210> 38

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HA epitope

<400> 38

Tyr Pro Tyr Asp Val Pro Asp Tyr Ala
1 5

<210> 39

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: KT3 epitope

<400> 39

ccaccagaac cagaaaca

18

<210> 40

<211> 6

<212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: KT3 epitope

<400> 40
 Pro Pro Glu Pro Glu Thr
 1 5

<210> 41
 <211> 36
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Myc epitope

<400> 41
 gcagaagaac aaaaattaat aagcgaagaa gactta

36

<210> 42
 <211> 12
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Myc epitope

<400> 42
 Ala Glu Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
 1 5 10

<210> 43
 <211> 717
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(717)

<220>
 <223> Description of Artificial Sequence: EYFP

<400> 43
 atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg 48
 Met Val Ser Lys Gly Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15

gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc 96
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30

gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc 144
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45


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tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc 192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
    50                      55                      60

ttc ggc tac ggc ctg cag tgc ttc gcc cgc tac ccc gac cac atg aag 240
Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys
    65                      70                      75                      80

cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag 288
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                      85                      90                      95

cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag 336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
                      100                      105                      110

gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc 384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
                      115                      120                      125

atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac 432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
                      130                      135                      140

aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac 480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
                      145                      150                      155                      160

ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc 528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                      165                      170                      175

gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc 576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
                      180                      185                      190

ccc gtg ctg ctg ccc gac aac cac tac ctg agc tac cag tcc gcc ctg 624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu
                      195                      200                      205

agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc 672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
                      210                      215                      220

gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag 717
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
                      225                      230                      235

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<210> 44

<211> 239

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: EYFP

<400> 44

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
  1                      5                      10                      15

```

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60
 Phe Gly Tyr Gly Leu Gln Cys Phe Ala Arg Tyr Pro Asp His Met Lys
 65 70 75 80
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
 165 170 175
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu
 195 200 205
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
 225 230 235

<210> 45
 <211> 717
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(717)

<220>
 <223> Description of Artificial Sequence: EGFP

<400> 45
 atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg 48
 Met Val Ser Lys Gly Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15
 gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc 96

Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly		
			20					25					30				
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144	
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile		
		35					40					45					
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192	
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr		
	50					55					60						
ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240	
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys		
	65				70				75						80		
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288	
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu		
			85						90					95			
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu		
			100					105					110				
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly		
		115					120					125					
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr		
	130					135					140						
aac	tac	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn		
	145				150					155					160		
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528	
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser		
			165					170				175					
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly		
			180				185						190				
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	acc	cag	tcc	gcc	ctg	624	
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu		
		195				200						205					
agc	aaa	gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	672	
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe		
	210					215					220						
gtg	acc	gcc	gcc	ggg	atc	act	ctc	ggc	atg	gac	gag	ctg	tac	aag		717	
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys			
	225				230					235							

<210> 46

<211> 239

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: EGFP

<400> 46

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1           5           10           15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
          20           25           30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
      35           40           45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
      50           55           60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
      65           70           75           80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
          85           90           95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
      100           105           110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
      115           120           125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
      130           135           140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
      145           150           155           160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
          165           170           175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
          180           185           190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
          195           200           205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
      210           215           220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
      225           230           235

```

<210> 47

<211> 717

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (1)..(717)

<220>

<223> Description of Artificial Sequence: EBFP

<400> 47

atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	gtg	gtg	ccc	atc	ctg	48
Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5					10					15		
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
		35					40					45				
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55					60					
ctg	acc	cac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	His	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70				75					80		
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
			85						90					95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
		115					120					125				
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
	130					135					140					
aac	ttc	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480
Asn	Phe	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	
145					150					155					160	
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
			165					170						175		
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180				185						190			
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	acc	cag	tcc	gcc	ctg	624
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	
		195					200					205				
agc	aaa	gac	ccc	aac	gag	aag	cgc	gat	cac	atg	gtc	ctg	ctg	gag	ttc	672
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	
	210					215					220					
gtg	acc	gcc	gcc	ggg	atc	act	ctc	ggc	atg	gac	gag	ctg	tac	aag		717
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys		
225					230					235						

<210> 48
 <211> 239
 <212> PRT
 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: EBFP

<400> 48

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1           5           10           15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
          20           25           30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
      35           40           45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
      50           55           60

Leu Thr His Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
      65           70           75           80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
          85           90           95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
          100          105          110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
      115          120          125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
      130          135          140

Asn Phe Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
      145          150          155          160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
          165          170          175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
          180          185          190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
          195          200          205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
      210          215          220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
      225          230          235

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<210> 49
 <211> 717
 <212> DNA
 <213> Artificial Sequence

<220>

<221> CDS

<222> (1) .. (717)

<220>

<223> Description of Artificial Sequence: ECFP

<400> 49

atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	gtg	gtg	ccc	atc	ctg	48
Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5					10					15		
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
		35					40					45				
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55					60					
ctg	acc	tgg	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Trp	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70				75						80	
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
				85					90					95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
		115					120					125				
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
	130					135					140					
aac	tac	atc	agc	cac	aac	gtc	tat	atc	acc	gcc	gac	aag	cag	aag	aac	480
Asn	Tyr	Ile	Ser	His	Asn	Val	Tyr	Ile	Thr	Ala	Asp	Lys	Gln	Lys	Asn	
145					150					155					160	
ggc	atc	aag	gcc	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
Gly	Ile	Lys	Ala	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
				165					170					175		
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180				185						190			
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	acc	cag	tcc	gcc	ctg	624
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	
		195														

agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc 672
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220

gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag 717
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
 225 230 235

<210> 50

<211> 239

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: ECFP

<400> 50

Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60

Leu Thr Trp Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140

Asn Tyr Ile Ser His Asn Val Tyr Ile Thr Ala Asp Lys Gln Lys Asn
 145 150 155 160

Gly Ile Lys Ala Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
 165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys

225

230

235

<210> 51
 <211> 720
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> CDS
 <222> (1)..(717)

<220>
 <223> Description of Artificial Sequence: Fred25

<400> 51
 atg gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt 48
 Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15
 gtt gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga 96
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30
 gag ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc 144
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45
 tgc act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act 192
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60
 ctg tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa 240
 Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80
 cgg cat gac ttt ttc aag agt gcc atg ccc gaa ggt tat gta cag gaa 288
 Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95
 agg acc atc ttc ttc aaa gat gac ggc aac tac aag aca cgt gct gaa 336
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110
 gtc aag ttt gaa ggt gat acc ctt gtt aat aga atc gag tta aaa ggt 384
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125
 att gac ttc aag gaa gat ggc aac att ctg gga cac aaa ttg gaa tac 432
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140
 aac tat aac tca cac aat gta tac atc atg gca gac aaa caa aag aat 480
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160
 gga atc aaa gtg aac ttc aag acc cgc cac aac att gaa gat gga agc 528
 Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
 165 170 175
 gtt caa cta gca gac cat tat caa caa aat act cca att ggc gat ggc 576

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Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
      180                      185                      190

cct gtc ctt tta cca gac aac cat tac ctg tcc aca caa tct gcc ctt 624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
      195                      200                      205

tcg aaa gat ccc aac gaa aag aga gac cac atg gtc ctt ctt gag ttt 672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
      210                      215                      220

gta aca gct gct ggg att aca cat ggc atg gat gaa ctg tac aac tag 720
Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn
      225                      230                      235

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<210> 52

<211> 239

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Fred25

<400> 52

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Met Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
  1              5              10              15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
      20              25              30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
      35              40              45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
      50              55              60

Leu Cys Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
      65              70              75              80

Arg His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
      85              90              95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
      100             105             110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
      115             120             125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
      130             135             140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
      145             150             155             160

Gly Ile Lys Val Asn Phe Lys Thr Arg His Asn Ile Glu Asp Gly Ser
      165             170             175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
      180                      185                      190

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Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220

Val Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Asn
 225 230 235

<210> 53

<211> 14

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Caspase-1,4,5
 substrate recognition sequence

<400> 53

tggaacatg acaa

14

<210> 54

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-1,4,5
 substrate recognition sequence

<400> 54

Trp Glu His Asp
 1

<210> 55

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-1
 substrate recognition sequence

<400> 55

tggtttaaag ac

12

<210> 56

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-1
 substrate recognition sequence

<400> 56

Trp Phe Lys Asp

1

<210> 57
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-2
substrate recognition sequence

<400> 57
gacgaacacg ac

12

<210> 58
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-2
substrate recognition sequence

<400> 58
Asp Glu His Asp
1

<210> 59
<211> 12
<212> DNA
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<220>
<223> Description of Artificial Sequence: Caspase-3,7
substrate recognition sequence

<400> 59
gacgaagttg ac

12

<210> 60
<211> 4
<212> PRT
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<220>
<223> Description of Artificial Sequence: Caspase-3,7
substrate recognition sequence

<400> 60
Asp Glu Val Asp
1

<210> 61
<211> 12
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-3
substrate recognition sequence

<400> 61

atagaaacag ac

12

<210> 62

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-3
substrate recognition sequence

<400> 62

Ile Glu Thr Asp

1

<210> 63

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-4,5
substrate recognition sequence

<400> 63

tgggtaagag ac

12

<210> 64

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-4,5
substrate recognition sequence

<400> 64

Trp Val Arg Asp

1

<210> 65

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-6
substrate recognition sequence

<400> 65

gtagaaatag ac

12

<210> 66
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<220>
<223> Description of Artificial Sequence: Caspase-6
substrate recognition sequence

<400> 66
Val Glu Ile Asp
1

<210> 67
<211> 12
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<220>
<223> Description of Artificial Sequence: Caspase-6
substrate recognition sequence

<400> 67
gtagaacacg ac

12

<210> 68
<211> 4
<212> PRT
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<220>
<223> Description of Artificial Sequence: Caspase-6
substrate recognition sequence

<400> 68
Val Glu His Asp
1

<210> 69
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: proCaspase-6
substrate recognition sequence

<400> 69
acagaagtag ac

12

<210> 70
<211> 4
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<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-6
substrate recognition sequence

<400> 70

Thr Glu Val Asp

1

<210> 71

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-7
substrate recognition sequence

<400> 71

atacaagcag ac

12

<210> 72

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-7
substrate recognition sequence

<400> 72

Ile Gln Ala Asp

1

<210> 73

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-8
substrate recognition sequence

<400> 73

gtagaaacag ac

12

<210> 74

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-8
substrate recognition sequence

<400> 74

Val Glu Thr Asp

1

<210> 75
<211> 12
<212> DNA
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<220>
<223> Description of Artificial Sequence: proCaspase-8
substrate recognition sequence

<400> 75
ttagaaacag ac

12

<210> 76
<211> 4
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<220>
<223> Description of Artificial Sequence: proCaspase-8
substrate recognition sequence

<400> 76
Leu Glu Thr Asp
1

<210> 77
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-9
substrate recognition sequence

<400> 77
ttagaacacg ac

12

<210> 78
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-9
substrate recognition sequence

<400> 78
Leu Glu His Asp
1

<210> 79
<211> 12
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-9
substrate recognition sequence

<400> 79

ttagaacacg ac

12

<210> 80

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-9
substrate recognition sequence

<400> 80

Leu Glu His Asp

1

<210> 81

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HIV protease
substrate recognition sequence

<400> 81

agccaaaatt ac

12

<210> 82

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HIV protease
substrate recognition sequence

<400> 82

Ser Gln Asn Tyr

1

<210> 83

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HIV protease
substrate recognition sequence

<400> 83

ccaatagtac aa

12

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-2
substrate recognition sequence

<400> 57

gacgaacacg ac

12

<210> 58

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-2
substrate recognition sequence

<400> 58

Asp Glu His Asp

1

<210> 59

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-3,7
substrate recognition sequence

<400> 59

gacgaagttg ac

12

<210> 60

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-3,7
substrate recognition sequence

<400> 60

Asp Glu Val Asp

1

<210> 61

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-3
substrate recognition sequence

<400> 61
atagaaacag ac

12

<210> 62
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
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substrate recognition sequence

<400> 62
Ile Glu Thr Asp
1

<210> 63
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: proCaspase-4,5
substrate recognition sequence

<400> 63
tgggtaagag ac

12

<210> 64
<211> 4
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: proCaspase-4,5
substrate recognition sequence

<400> 64
Trp Val Arg Asp
1

<210> 65
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-6
substrate recognition sequence

<400> 65
gtagaaatag ac

12

<210> 66
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<220>
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substrate recognition sequence

<400> 66
Val Glu Ile Asp
1

<210> 67
<211> 12
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-6
substrate recognition sequence

<400> 67
gtagaacacg ac

12

<210> 68
<211> 4
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-6
substrate recognition sequence

<400> 68
Val Glu His Asp
1

<210> 69
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: proCaspase-6
substrate recognition sequence

<400> 69
acagaagtag ac

12

<210> 70
<211> 4
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: proCaspase-6
substrate recognition sequence

<400> 70
Thr Glu Val Asp
1

<210> 71
<211> 12
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<220>
<223> Description of Artificial Sequence: proCaspase-7
substrate recognition sequence

<400> 71
atacaagcag ac

12

<210> 72
<211> 4
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substrate recognition sequence

<400> 72
Ile Gln Ala Asp
1

<210> 73
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-8
substrate recognition sequence

<400> 73
gtagaaacag ac

12

<210> 74
<211> 4

<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-8
substrate recognition sequence

<400> 74
Val Glu Thr Asp
1

<210> 75
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: proCaspase-8
substrate recognition sequence

<400> 75
ttagaaacag ac

12

<210> 76
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: proCaspase-8
substrate recognition sequence

<400> 76
Leu Glu Thr Asp
1

<210> 77
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-9
substrate recognition sequence

<400> 77
ttagaacacg ac

12

<210> 78
<211> 4
<212> PRT
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Caspase-9
substrate recognition sequence

<400> 78

Leu Glu His Asp

1

<210> 79

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: proCaspase-9
substrate recognition sequence

<400> 79

ttagaacacg ac

12

<210> 80

<211> 4

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence: proCaspase-9
substrate recognition sequence

<400> 80

Leu Glu His Asp

1

<210> 81

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HIV protease
substrate recognition sequence

<400> 81

agccaaaatt ac

12

<210> 82

<211> 4

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HIV protease
substrate recognition sequence

<400> 82
Ser Gln Asn Tyr
1

<210> 83
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: HIV protease
substrate recognition sequence

<400> 83
ccaatagtag aa

12

<210> 84
<211> 4
<212> PRT
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<220>
<223> Description of Artificial Sequence: HIV protease
substrate recognition sequence

<400> 84
Pro Ile Val Gln
1

<210> 85
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Adenovirus
endopeptidase substrate recognition sequence

<400> 85
atgttttggag ga

12

<210> 86
<211> 4
<212> PRT
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<220>
<223> Description of Artificial Sequence: Adenovirus
endopeptidase substrate recognition sequence

<400> 86
Met Phe Gly Gly

1

<210> 87
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Adenovirus
 endopeptidase substrate recognition sequence

<400> 87
gcaaaaaaaaa ga

12

<210> 88
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Adenovirus
 endopeptidase substrate recognition sequence

<400> 88
Ala Lys Lys Arg
1

<210> 89
<211> 9
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: b-Secretase
 substrate recognition sequence

<400> 89
gtgaaaatg

9

<210> 90
<211> 3
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: b-Secretase
 substrate recognition sequence

<400> 90
Val Lys Met
1

<210> 91
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: b-Secretase
substrate recognition sequence

<400> 91
gacgcagaat tc

12

<210> 92
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: b-Secretase
substrate recognition sequence

<400> 92
Asp Ala Glu Phe
1

<210> 93
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Cathepsin D
substrate recognition sequence

<400> 93
aaaccagcat tattc

15

<210> 94
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Cathepsin D
substrate recognition sequence

<400> 94
Lys Pro Ala Leu Phe
1 5

<210> 95
<211> 9
<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Cathepsin D
substrate recognition sequence

<400> 95

ttcagatta

9

<210> 96

<211> 3

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Cathepsin D
substrate recognition sequence

<400> 96

Phe Arg Leu

1

<210> 97

<211> 15

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Matrix
Metalloprotease substrate recognition sequence

<400> 97

ggaccattag gacca

15

<210> 98

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Matrix
Metalloprotease substrate recognition sequence

<400> 98

Gly Pro Leu Gly Pro

1

5

<210> 99

<211> 12

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Granzyme B
substrate recognition sequence

<400> 99
atagaaccag ac

12

<210> 100
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Granzyme B
substrate recognition sequence

<400> 100
Ile Glu Pro Asp
1

<210> 101
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Anthrax
protease substrate recognition sequence

<400> 101
atgcccaaga agaagccgac gcccatccag ctgaac

36

<210> 102
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Anthrax
protease substrate recognition sequence

<400> 102
Met Pro Lys Lys Lys Pro Thr Pro Ile Gln Leu Asn
1 5 10

<210> 103
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Anthrax
protease substrate recognition sequence

<400> 103
atgctggccc ggaggaagcc ggtgctgccg gcgctcacca tcaac 45

<210> 104
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Anthrax
protease substrate recognition sequence

<400> 104
Met Leu Ala Arg Arg Lys Pro Val Leu Pro Ala Leu Thr Ile Asn
1 5 10 15

<210> 105
<211> 18
<212> DNA
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<220>
<223> Description of Artificial Sequence:
tetanus/botulium substrate recognition sequence

<400> 105
gcctcgcagt ttgaaaca 18

<210> 106
<211> 6
<212> PRT
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<220>
<223> Description of Artificial Sequence:
tetanus/botulium substrate recognition sequence

<400> 106
Ala Ser Gln Phe Glu Thr
1 5

<210> 107
<211> 18
<212> DNA
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<220>
<223> Description of Artificial Sequence:
tetanus/botulium substrate recognition sequence

<400> 107
gcttctcaat ttgaaacg 18

<210> 108
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
tetanus/botulium substrate recognition sequence

<400> 108
Ala Ser Gln Phe Glu Thr
1 5

<210> 109
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Botulinum
neurotoxin A substrate recognition sequence

<400> 109
gcccaaccaac gtgcaaca

18

<210> 110
<211> 6
<212> PRT
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<220>
<223> Description of Artificial Sequence: Botulinum
neurotoxin A substrate recognition sequence

<400> 110
Ala Asn Gln Arg Ala Thr
1 5

<210> 111
<211> 18
<212> DNA
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<220>
<223> Description of Artificial Sequence: Botulinum
neurotoxin B substrate recognition sequence

<400> 111
gcttctcaat ttgaaacg

18

<210> 112
<211> 6

<212> PRT
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<220>
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neurotoxin B substrate recognition sequence

<400> 112
Ala Ser Gln Phe Glu Thr
1 5

<210> 113
<211> 18
<212> DNA
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<220>
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neurotoxin C substrate recognition sequence

<400> 113
acgaaaaaag ctgtgaaa

18

<210> 114
<211> 6
<212> PRT
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<220>
<223> Description of Artificial Sequence: Botulinum
neurotoxin C substrate recognition sequence

<400> 114
Thr Lys Lys Ala Val Lys
1 5

<210> 115
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Botulinum
neurotoxin D substrate recognition sequence

<400> 115
gaccagaagc tctctgag

18

<210> 116
<211> 6
<212> PRT
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Botulinum
neurotoxin D substrate recognition sequence

<400> 116

Asp Gln Lys Leu Ser Glu
1 5

<210> 117

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Botulinum
neurotoxin E substrate recognition sequence

<400> 117

atcgacagga tcatggag

18

<210> 118

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Botulinum
neurotoxin E substrate recognition sequence

<400> 118

Ile Asp Arg Ile Met Glu
1 5

<210> 119

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Botulinum
neurotoxin F substrate recognition sequence

<400> 119

agagaccaga agctctct

18

<210> 120

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Botulinum
neurotoxin F substrate recognition sequence

<400> 120
Arg Asp Gln Lys Leu Ser
1 5

<210> 121
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Botulinum
neurotoxin G substrate recognition sequence

<400> 121
acgagcgcag ccaagttg 18

<210> 122
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Botulinum
neurotoxin G substrate recognition sequence

<400> 122
Thr Ser Ala Ala Lys Leu
1 5

<210> 123
<211> 69
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Cytoplasm/cytoskeleton target sequence

<400> 123
atgtctactg tccacgaaat cctgtgcaag ctcagcttgg aggggtgttca ttctacaccc 60
ccaagtgcc 69

<210> 124
<211> 23
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
Cytoplasm/cytoskeleton target sequence

<400> 124

Met Ser Thr Val His Glu Ile Leu Cys Lys Leu Ser Leu Glu Gly Val
 1 5 10 15

His Ser Thr Pro Pro Ser Ala
 20

<210> 125

<211> 96

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Inner surface
 of plasma membrane target sequence

<400> 125

atgggatgta cattaagcgc agaagacaaa gcagcagtag aaagaagcaa aatgatagac 60
 agaaaacttaa gagaagacgg agaaaaagct gctaga 96

<210> 126

<211> 32

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Inner surface
 of plasma membrane target sequence

<400> 126

Met Gly Cys Thr Leu Ser Ala Glu Asp Lys Ala Ala Val Glu Arg Ser
 1 5 10 15

Lys Met Ile Asp Arg Asn Leu Arg Glu Asp Gly Glu Lys Ala Ala Arg
 20 25 30

<210> 127

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Nucleus target
 sequence

<400> 127

agaaggaaac gacaaaag

18

<210> 128

<211> 6
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Nucleus target
 sequence

<400> 128
 Arg Arg Lys Arg Gln Lys
 1 5

<210> 129
 <211> 90
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Nucleolus
 target sequence

<400> 129
 agaaaacgta tacgtactta cctcaagtcc tgcaggcgga tgaaaagaag tggttttgag 60
 atgtctcgac ctattccttc ccaccttact 90

<210> 130
 <211> 30
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Nucleolus
 target sequence

<400> 130
 Arg Lys Arg Ile Arg Thr Tyr Leu Lys Ser Cys Arg Arg Met Lys Arg
 1 5 10 15

Ser Gly Phe Glu Met Ser Arg Pro Ile Pro Ser His Leu Thr
 20 25 30

<210> 131
 <211> 87
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Mitochondria
 target sequence

<400> 131
 atgtccgtcc tgacgccgct gctgctgcgg ggcttgacag gctcggcccg gcggctccca 60

gtgccgcgcgcg ccaagatcca ttcgttg

87

<210> 132

<211> 29

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Mitochondria
target sequence

<400> 132

Met Ser Val Leu Thr Pro Leu Leu Leu Arg Gly Leu Thr Gly Ser Ala
1 5 10 15

Arg Arg Leu Pro Val Pro Arg Ala Leu Ile His Ser Leu
20 25

<210> 133

<211> 99

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Nuclear
Envelope target sequence

<400> 133

atgagcattg ttttaataat tggtattgtg gtgatttttt taatatgttt tttatattta 60
agcaacagca aagatcccag agtaccagtt gaattaatg 99

<210> 134

<211> 33

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Nuclear
Envelope target sequence

<400> 134

Met Ser Ile Val Leu Ile Ile Val Ile Val Val Ile Phe Leu Ile Cys
1 5 10 15

Phe Leu Tyr Leu Ser Asn Ser Lys Asp Pro Arg Val Pro Val Glu Leu
20 25 30

Met

<210> 135

<211> 246

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Golgi target sequence

<400> 135

atgaggcttc gggagccgct cctgagcggc agcgccgcga tgccaggcgc gtccttacag 60
 cgggectgcc gcctgctcgt ggccgtctgc gctctgcacc ttggcgtcac cctcgtttac 120
 tacctggctg gccgcgacct gagccgcctg cccaactgg tcggagtctc cacaccgctg 180
 cagggcggct cgaacagtgc cgccgccatc gggcagtcct ccggggagct ccggaccgga 240
 ggggcc 246

<210> 136

<211> 82

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Golgi target sequence

<400> 136

Met Arg Leu Arg Glu Pro Leu Leu Ser Gly Ser Ala Ala Met Pro Gly
 1 5 10 15
 Ala Ser Leu Gln Arg Ala Cys Arg Leu Leu Val Ala Val Cys Ala Leu
 20 25 30
 His Leu Gly Val Thr Leu Val Tyr Tyr Leu Ala Gly Arg Asp Leu Ser
 35 40 45
 Arg Leu Pro Gln Leu Val Gly Val Ser Thr Pro Leu Gln Gly Gly Ser
 50 55 60
 Asn Ser Ala Ala Ala Ile Gly Gln Ser Ser Gly Glu Leu Arg Thr Gly
 65 70 75 80
 Gly Ala

<210> 137

<211> 150

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Endoplasmic reticulum target sequence

<400> 137
 gaaacaataa gacctataag aataagaaga tggtcttatt ttacatctac agacagcaaa 60
 atggcaattc aattaagatc tccctttcca ttagcattac caggaatgtt agctttatta 120
 ggatggtggt ggtttttcag tagaaaaaaa 150

<210> 138
 <211> 50
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Endoplasmic
 reticulum target sequence

<400> 138
 Glu Thr Ile Arg Pro Ile Arg Ile Arg Arg Cys Ser Tyr Phe Thr Ser
 1 5 10 15
 Thr Asp Ser Lys Met Ala Ile Gln Leu Arg Ser Pro Phe Pro Leu Ala
 20 25 30
 Leu Pro Gly Met Leu Ala Leu Leu Gly Trp Trp Trp Phe Phe Ser Arg
 35 40 45
 Lys Lys
 50

<210> 139
 <211> 39
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Nuclear Export
 target sequence

<400> 139
 gccttgcaaga agaagctgga ggagctagag cttgatgag 39

<210> 140
 <211> 13
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Nuclear Export
 target sequence

<400> 140
 Ala Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Asp Glu
 1 5 10

<210> 141
 <211> 1024
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Size exclusion
 target sequence

<400> 141
 gccgacctca gtcttgtgga tgcgttgaca gaaccacctc cagaaattga gggagaaata 60
 aagcgagact tcatggctgc gctggaggca gagccctatg atgacatcgt gggagaaact 120
 gtggagaaaa ctgagtttat tcctctcctg gatggtgatg agaaaaccgg gaactcagag 180
 tccaaaaaga aaccctgctt agacactagc cagggtgaag gtatcccatc ttctaaacca 240
 acactcctag ccaatgggta tcatggaatg gaggggaata aactgcagg gtctccaact 300
 gacttccttg aagagagagt ggactatccg gattatcaga gcagccagaa ctggccagaa 360
 gatgcaagct tttgtttcca gcctcagcaa gtgtagata ctgaccaggc tgagcccttt 420
 aacgagcacc gtgatgatgg tttggcagat ctgctctttg tctccagtgg acccacgaac 480
 gcttctgcat ttacagagcg agacaatcct tcagaagaca gttacggtat gcttcctgt 540
 gactcatttg cttccacggc tgttgatatc caggagtggc ctgtgggagc cccaaactct 600
 ccatgttcag agtctgtgt ctccccagag gttactatag aaacctaca gccagcaaca 660
 gagctctcca aggcagcaga agtggaatca gtgaaagagc agctgccagc taaagcattg 720
 gaaacgatgg cagagcagac cactgatgtg gtgcactctc catccacaga cacaacacca 780
 ggcccagaca cagaggcagc actggctaaa gacatagaag agatcaccaa gccagatgtg 840
 atattggcaa atgtcacgca gccatctact gaatcggata tggtcctggc ccaggacatg 900
 gaactactca caggaacaga ggcagcccac gctaacaata tcatattgcc tacagaacca 960
 gacgaatctt caaccaagga tgtagacca cctatggaag aagaaattgt ccaggcaat 1020
 gata 1024

<210> 142
 <211> 566
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Size exclusion
 target sequence

<400> 142
 Ala Asp Leu Ser Leu Val Asp Ala Leu Thr Glu Pro Pro Pro Glu Ile
 1 5 10 15
 Glu Gly Glu Ile Lys Arg Asp Phe Met Ala Ala Leu Glu Ala Glu Pro
 20 25 30
 Tyr Asp Asp Ile Val Gly Glu Thr Val Glu Lys Thr Glu Phe Ile Pro
 35 40 45
 Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn Ser Glu Ser Lys Lys Lys
 50 55 60
 Pro Cys Leu Asp Thr Ser Gln Val Glu Gly Ile Pro Ser Ser Lys Pro
 65 70 75 80
 Thr Leu Leu Ala Asn Gly Asp His Gly Met Glu Gly Asn Asn Thr Ala
 85 90 95
 Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg Val Asp Tyr Pro Asp Tyr
 100 105 110
 Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala Ser Phe Cys Phe Gln Pro
 115 120 125
 Gln Gln Val Leu Asp Thr Asp Gln Ala Glu Pro Phe Asn Glu His Arg
 130 135 140
 Asp Asp Gly Leu Ala Asp Leu Leu Phe Val Ser Ser Gly Pro Thr Asn
 145 150 155 160
 Ala Ser Ala Phe Thr Glu Arg Asp Asn Pro Ser Glu Asp Ser Tyr Gly
 165 170 175
 Met Leu Pro Cys Asp Ser Phe Ala Ser Thr Ala Val Val Ser Gln Glu
 180 185 190
 Trp Ser Val Gly Ala Pro Asn Ser Pro Cys Ser Glu Ser Cys Val Ser
 195 200 205
 Pro Glu Val Thr Ile Glu Thr Leu Gln Pro Ala Thr Glu Leu Ser Lys
 210 215 220
 Ala Ala Glu Val Glu Ser Val Lys Glu Gln Leu Pro Ala Lys Ala Leu
 225 230 235 240
 Glu Thr Met Ala Glu Gln Thr Thr Asp Val Val His Ser Pro Ser Thr
 245 250 255
 Asp Thr Thr Pro Gly Pro Asp Thr Glu Ala Ala Leu Ala Lys Asp Ile
 260 265 270
 Glu Glu Ile Thr Lys Pro Asp Val Ile Leu Ala Asn Val Thr Gln Pro
 275 280 285
 Ser Thr Glu Ser Asp Met Phe Leu Ala Gln Asp Met Glu Leu Leu Thr
 290 295 300

Gly Thr Glu Ala Ala His Ala Asn Asn Ile Ile Leu Pro Thr Glu Pro
 305 310 315 320
 Asp Glu Ser Ser Thr Lys Asp Val Ala Pro Pro Met Glu Glu Glu Ile
 325 330 335
 Val Pro Gly Asn Asp Thr Thr Ser Pro Lys Glu Thr Glu Thr Thr Leu
 340 345 350
 Pro Ile Lys Met Asp Leu Ala Pro Pro Glu Asp Val Leu Leu Thr Lys
 355 360 365
 Glu Thr Glu Leu Ala Pro Ala Lys Gly Met Val Ser Leu Ser Glu Ile
 370 375 380
 Glu Glu Ala Leu Ala Lys Asn Asp Val Arg Ser Ala Glu Ile Pro Val
 385 390 395 400
 Ala Gln Glu Thr Val Val Ser Glu Thr Glu Val Val Leu Ala Thr Glu
 405 410 415
 Val Val Leu Pro Ser Asp Pro Ile Thr Thr Leu Thr Lys Asp Val Thr
 420 425 430
 Leu Pro Leu Glu Ala Glu Arg Pro Leu Val Thr Asp Met Thr Pro Ser
 435 440 445
 Leu Glu Thr Glu Met Thr Leu Gly Lys Glu Thr Ala Pro Pro Thr Glu
 450 455 460
 Thr Asn Leu Gly Met Ala Lys Asp Met Ser Pro Leu Pro Glu Ser Glu
 465 470 475 480
 Val Thr Leu Gly Lys Asp Val Val Ile Leu Pro Glu Thr Lys Val Ala
 485 490 495
 Glu Phe Asn Asn Val Thr Pro Leu Ser Glu Glu Glu Val Thr Ser Val
 500 505 510
 Lys Asp Met Ser Pro Ser Ala Glu Thr Glu Ala Pro Leu Ala Lys Asn
 515 520 525
 Ala Asp Leu His Ser Gly Thr Glu Leu Ile Val Asp Asn Ser Met Ala
 530 535 540
 Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu Thr Lys Val Ala Thr Val
 545 550 555 560
 Pro Ile Lys Asp Lys Gly
 565

<210> 143

<211> 63

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Vesicle
membrane target sequence

<400> 143

atgtggggcaa tcgggattac tgttctgggt atcttcatca tcatcatcat cgtgtggggt 60
gtc 63

<210> 144

<211> 21

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Vesicle
membrane target sequence

<400> 144

Met Trp Ala Ile Gly Ile Thr Val Leu Val Ile Phe Ile Ile Ile Ile
1 5 10 15
Ile Val Trp Val Val
20

<210> 145

<211> 61

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Vesicle
membrane target sequence

<400> 145

atgtggggcga tagggatcag tgtcctgggt atcattgtca tcatcatcat cgtgtgggtgt 60
g 61

<210> 146

<211> 20

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Vesicle
membrane target sequence

<400> 146

Met Trp Ala Ile Gly Ile Ser Val Leu Val Ile Ile Val Ile Ile Ile
1 5 10 15
Ile Val Trp Cys

20

<210> 147
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Nuclear Export
target sequence

<400> 147
gacctgcaga agaagctgga ggagctggaa cttgacgag

39

<210> 148
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Nuclear Export
target sequence

<400> 148
Asp Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Asp Glu
1 5 10

<210> 149
<211> 9
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Peroxisome
target sequence

<400> 149
tctaaactg

9

<210> 150
<211> 3
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Peroxisome
target sequence

<400> 150
Ser Lys Leu
1

<210> 151
 <211> 3378
 <212> DNA
 <213> Mus musculus

<220>
 <221> CDS
 <222> (1)..(3375)

<400> 151
 atg gcc gac ctc agt ctt gtg gat gcg ttg aca gaa cca cct cca gaa 48
 Met Ala Asp Leu Ser Leu Val Asp Ala Leu Thr Glu Pro Pro Pro Glu
 1 5 10 15
 att gag gga gaa ata aag cga gac ttc atg gct gcg ctg gag gca gag 96
 Ile Glu Gly Glu Ile Lys Arg Asp Phe Met Ala Ala Leu Glu Ala Glu
 20 25 30
 ccc tat gat gac atc gtg gga gaa act gtg gag aaa act gag ttt att 144
 Pro Tyr Asp Asp Ile Val Gly Glu Thr Val Glu Lys Thr Glu Phe Ile
 35 40 45
 cct ctc ctg gat ggt gat gag aaa acc ggg aac tca gag tcc aaa aag 192
 Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn Ser Glu Ser Lys Lys
 50 55 60
 aaa ccc tgc tta gac act agc cag gtt gaa ggt atc cca tct tct aaa 240
 Lys Pro Cys Leu Asp Thr Ser Gln Val Glu Gly Ile Pro Ser Ser Lys
 65 70 75 80
 cca aca ctc cta gcc aat ggt gat cat gga atg gag ggg aat aac act 288
 Pro Thr Leu Leu Ala Asn Gly Asp His Gly Met Glu Gly Asn Asn Thr
 85 90 95
 gca ggg tct cca act gac ttc ctt gaa gag aga gtg gac tat ccg gat 336
 Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg Val Asp Tyr Pro Asp
 100 105 110
 tat cag agc agc cag aac tgg cca gaa gat gca agc ttt tgt ttc cag 384
 Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala Ser Phe Cys Phe Gln
 115 120 125
 cct cag caa gtg tta gat act gac cag gct gag ccc ttt aac gag cac 432
 Pro Gln Gln Val Leu Asp Thr Asp Gln Ala Glu Pro Phe Asn Glu His
 130 135 140
 cgt gat gat ggt ttg gca gat ctg ctc ttt gtc tcc agt gga ccc acg 480
 Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe Val Ser Ser Gly Pro Thr
 145 150 155 160
 aac gct tct gca ttt aca gag cga gac aat cct tca gaa gac agt tac 528
 Asn Ala Ser Ala Phe Thr Glu Arg Asp Asn Pro Ser Glu Asp Ser Tyr
 165 170 175
 ggt atg ctt ccc tgt gac tca ttt gct tcc acg gct gtt gta tct cag 576
 Gly Met Leu Pro Cys Asp Ser Phe Ala Ser Thr Ala Val Val Ser Gln
 180 185 190

gag tgg tct gtg gga gcc cca aac tct cca tgt tca gag tcc tgt gtc Glu Trp Ser Val Gly Ala Pro Asn Ser Pro Cys Ser Glu Ser Cys Val	624
195 200 205	
tcc cca gag gtt act ata gaa acc cta cag cca gca aca gag ctc tcc Ser Pro Glu Val Thr Ile Glu Thr Leu Gln Pro Ala Thr Glu Leu Ser	672
210 215 220	
aag gca gca gaa gtg gaa tca gtg aaa gag cag ctg cca gct aaa gca Lys Ala Ala Glu Val Ser Val Lys Glu Gln Leu Pro Ala Lys Ala	720
225 230 235 240	
ttg gaa acg atg gca gag cag acc act gat gtg gtg cac tct cca tcc Leu Glu Thr Met Ala Glu Gln Thr Thr Asp Val Val His Ser Pro Ser	768
245 250 255	
aca gac aca aca cca ggc cca gac aca gag gca gca ctg gct aaa gac Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu Ala Ala Leu Ala Lys Asp	816
260 265 270	
ata gaa gag atc acc aag cca gat gtg ata ttg gca aat gtc acg cag Ile Glu Glu Ile Thr Lys Pro Asp Val Ile Leu Ala Asn Val Thr Gln	864
275 280 285	
cca tct act gaa tcg gat atg ttc ctg gcc cag gac atg gaa cta ctc Pro Ser Thr Glu Ser Asp Met Phe Leu Ala Gln Asp Met Glu Leu Leu	912
290 295 300	
aca gga aca gag gca gcc cac gct aac aat atc ata ttg cct aca gaa Thr Gly Thr Glu Ala Ala His Ala Asn Asn Ile Ile Leu Pro Thr Glu	960
305 310 315 320	
cca gac gaa tct tca acc aag gat gta gca cca cct atg gaa gaa gaa Pro Asp Glu Ser Thr Lys Asp Val Ala Pro Pro Met Glu Glu Glu	1008
325 330 335	
att gtc cca ggc aat gat acg aca tcc ccc aaa gaa aca gag aca aca Ile Val Pro Gly Asn Asp Thr Thr Ser Pro Lys Glu Thr Glu Thr Thr	1056
340 345 350	
ctt cca ata aaa atg gac ttg gca cca cct gag gat gtg tta ctt acc Leu Pro Ile Lys Met Asp Leu Ala Pro Pro Glu Asp Val Leu Leu Thr	1104
355 360 365	
aaa gaa aca gaa cta gcc cca gcc aag ggc atg gtt tca ctc tca gaa Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly Met Val Ser Leu Ser Glu	1152
370 375 380	
ata gaa gag gct ctg gca aag aat gat gtt cgc tct gca gaa ata cct Ile Glu Glu Ala Leu Lys Asn Asp Val Arg Ser Ala Glu Ile Pro	1200
385 390 395 400	
gtg gct cag gag aca gtg gtc tca gaa aca gag gtg gtc ctg gca aca Val Ala Gln Glu Thr Val Val Ser Glu Thr Glu Val Val Leu Ala Thr	1248
405 410 415	

gaa gtg gta ctg ccc tca gat ccc ata aca aca ttg aca aag gat gtg Glu Val Val Leu Pro Ser Asp Pro Ile Thr Thr Leu Thr Lys Asp Val 420 425 430	1296
aca ctc ccc tta gaa gca gag aga ccg ttg gtg acg gac atg act cca Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu Val Thr Asp Met Thr Pro 435 440 445	1344
tct ctg gaa aca gaa atg acc cta ggc aaa gag aca gct cca ccc aca Ser Leu Glu Thr Glu Met Thr Leu Gly Lys Glu Thr Ala Pro Pro Thr 450 455 460	1392
gaa aca aat ttg ggc atg gcc aaa gac atg tct cca ctc cca gaa tca Glu Thr Asn Leu Gly Met Ala Lys Asp Met Ser Pro Leu Pro Glu Ser 465 470 475 480	1440
gaa gtg act ctg ggc aag gac gtg gtt ata ctt cca gaa aca aag gtg Glu Val Thr Leu Gly Lys Asp Val Val Ile Leu Pro Glu Thr Lys Val 485 490 495	1488
gct gag ttt aac aat gtg act cca ctt tca gaa gaa gag gta acc tca Ala Glu Phe Asn Asn Val Thr Pro Leu Ser Glu Glu Glu Val Thr Ser 500 505 510	1536
gtc aag gac atg tct ccg tct gca gaa aca gag gct ccc ctg gct aag Val Lys Asp Met Ser Pro Ser Ala Glu Thr Glu Ala Pro Leu Ala Lys 515 520 525	1584
aat gct gat ctg cac tca gga aca gag ctg att gtg gac aac agc atg Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile Val Asp Asn Ser Met 530 535 540	1632
gct cca gcc tcc gat ctt gca ctg ccc ttg gaa aca aaa gta gca aca Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu Thr Lys Val Ala Thr 545 550 555 560	1680
gtt cca att aaa gac aaa gga act gta cag act gaa gaa aaa cca cgt Val Pro Ile Lys Asp Lys Gly Thr Val Gln Thr Glu Glu Lys Pro Arg 565 570 575	1728
gaa gac tcc cag tta gca tct atg cag cac aag gga cag tca aca gta Glu Asp Ser Gln Leu Ala Ser Met Gln His Lys Gly Gln Ser Thr Val 580 585 590	1776
cct cct tgc acg gct tca cca gaa cca gtc aaa gct gca gaa caa atg Pro Pro Cys Thr Ala Ser Pro Glu Pro Val Lys Ala Ala Glu Gln Met 595 600 605	1824
tct acc tta cca ata gat gca cct tct cca tta gag aac tta gag cag Ser Thr Leu Pro Ile Asp Ala Pro Ser Pro Leu Glu Asn Leu Glu Gln 610 615 620	1872
aag gaa acg cct ggc agc cag cct tct gag cct tgc tca gga gta tcc Lys Glu Thr Pro Gly Ser Gln Pro Ser Glu Pro Cys Ser Gly Val Ser 625 630 635 640	1920
cgg caa gaa gaa gca aag gct gct gta ggt gtg act gga aat gac atc	1968

Arg	Gln	Glu	Glu	Ala	Lys	Ala	Ala	Val	Gly	Val	Thr	Gly	Asn	Asp	Ile	
				645					650					655		
act	acc	ccg	cca	aac	aag	gag	cca	cca	cca	agc	cca	gaa	aag	aaa	gca	2016
Thr	Thr	Pro	Pro	Asn	Lys	Glu	Pro	Pro	Pro	Ser	Pro	Glu	Lys	Lys	Ala	
			660					665					670			
aag	cct	ttg	gcc	acc	act	caa	cct	gca	aag	act	tca	aca	tcg	aaa	gcc	2064
Lys	Pro	Leu	Ala	Thr	Thr	Gln	Pro	Ala	Lys	Thr	Ser	Thr	Ser	Lys	Ala	
		675					680					685				
aaa	aca	cag	ccc	act	tct	ctc	cct	aag	caa	cca	gct	ccc	acc	acc	tct	2112
Lys	Thr	Gln	Pro	Thr	Ser	Leu	Pro	Lys	Gln	Pro	Ala	Pro	Thr	Thr	Ser	
		690				695					700					
ggg	ggg	ttg	aat	aaa	aaa	ccc	atg	agc	ctc	gcc	tca	ggc	tca	gtg	cca	2160
Gly	Gly	Leu	Asn	Lys	Lys	Pro	Met	Ser	Leu	Ala	Ser	Gly	Ser	Val	Pro	
705					710					715					720	
gct	gcc	cca	cac	aaa	cgc	cct	gct	gct	gcc	act	gct	act	gcc	agg	cct	2208
Ala	Ala	Pro	His	Lys	Arg	Pro	Ala	Ala	Ala	Thr	Ala	Thr	Ala	Arg	Pro	
				725					730					735		
tcc	acc	cta	cct	gcc	aga	gac	gtg	aag	cca	aag	cca	att	aca	gaa	gct	2256
Ser	Thr	Leu	Pro	Ala	Arg	Asp	Val	Lys	Pro	Lys	Pro	Ile	Thr	Glu	Ala	
			740					745					750			
aag	gtt	gcc	gaa	aag	cgg	acc	tct	cca	tcc	aag	cct	tca	tct	gcc	cca	2304
Lys	Val	Ala	Glu	Lys	Arg	Thr	Ser	Pro	Ser	Lys	Pro	Ser	Ser	Ala	Pro	
		755					760					765				
gcc	ctc	aaa	cct	gga	cct	aaa	acc	acc	cca	acc	gtt	tca	aaa	gcc	aca	2352
Ala	Leu	Lys	Pro	Gly	Pro	Lys	Thr	Thr	Pro	Thr	Val	Ser	Lys	Ala	Thr	
	770					775					780					
tct	ccc	tca	act	ctt	gtt	tcc	act	gga	cca	agt	agt	aga	agt	cca	gct	2400
Ser	Pro	Ser	Thr	Leu	Val	Ser	Thr	Gly	Pro	Ser	Ser	Arg	Ser	Pro	Ala	
785					790					795					800	
aca	act	ctg	cct	aag	agg	cca	acc	agc	atc	aag	act	gag	ggg	aaa	cct	2448
Thr	Thr	Leu	Pro	Lys	Arg	Pro	Thr	Ser	Ile	Lys	Thr	Glu	Gly	Lys	Pro	
				805					810					815		
gct	gat	gtc	aaa	agg	atg	act	gct	aag	tct	gcc	tca	gct	gac	ttg	agt	2496
Ala	Asp	Val	Lys	Arg	Met	Thr	Ala	Lys	Ser	Ala	Ser	Ala	Asp	Leu	Ser	
			820					825					830			
cgc	tca	aag	acc	acc	tct	gcc	agt	tct	gtg	aag	aga	aac	acc	act	ccc	2544
Arg	Ser	Lys	Thr	Thr	Ser	Ala	Ser	Ser	Val	Lys	Arg	Asn	Thr	Thr	Pro	
		835					840					845				
act	ggg	gca	gca	ccc	cca	gca	ggg	atg	act	tcc	act	cga	gtc	aag	ccc	2592
Thr	Gly	Ala	Ala	Pro	Pro	Ala	Gly	Met	Thr	Ser	Thr	Arg	Val	Lys	Pro	
	850					855					860					
atg	tct	gca	cct	agc	cgc	tct	tct	ggg	gct	ctt	tct	gtg	gac	aag	aag	2640
Met	Ser	Ala	Pro	Ser	Arg	Ser	Ser	Gly	Ala	Leu	Ser	Val	Asp	Lys	Lys	

865	870	875	880	
ccc act tcc act aag cct agc tcc tct gct ccc agg gtg agc cgc ctg				2688
Pro Thr Ser Thr Lys Pro Ser Ser Ser Ala Pro Arg Val Ser Arg Leu				
885		890	895	
gcc aca act gtt tct gcc cct gac ctg aag agt gtt cgc tcc aag gtc				2736
Ala Thr Thr Val Ser Ala Pro Asp Leu Lys Ser Val Arg Ser Lys Val				
900		905	910	
ggc tct aca gaa aac atc aaa cac cag cct gga gga ggc cgg gcc aaa				2784
Gly Ser Thr Glu Asn Ile Lys His Gln Pro Gly Gly Gly Arg Ala Lys				
915		920	925	
gta gag aaa aaa aca gag gca gct acc aca gct ggg aag cct gaa cct				2832
Val Glu Lys Lys Thr Glu Ala Ala Thr Thr Ala Gly Lys Pro Glu Pro				
930		935	940	
aat gca gtc act aaa gca gcc ggc tcc att gcg agt gca cag aaa ccg				2880
Asn Ala Val Thr Lys Ala Ala Gly Ser Ile Ala Ser Ala Gln Lys Pro				
945		950	955	960
cct gct ggg aaa gtc cag ata gta tcc aaa aaa gtg agc tac agt cat				2928
Pro Ala Gly Lys Val Gln Ile Val Ser Lys Lys Val Ser Tyr Ser His				
965		970	975	
att caa tcc aag tgt gtt tcc aag gac aat att aag cat gtc cct gga				2976
Ile Gln Ser Lys Cys Val Ser Lys Asp Asn Ile Lys His Val Pro Gly				
980		985	990	
tgt ggc aat gtt cag att cag aac aag aaa gtg gac ata tcc aag gtc				3024
Cys Gly Asn Val Gln Ile Gln Asn Lys Lys Val Asp Ile Ser Lys Val				
995		1000	1005	
tcc tcc aag tgt ggg tcc aaa gct aat atc aag cac aag cct ggt gga				3072
Ser Ser Lys Cys Gly Ser Lys Ala Asn Ile Lys His Lys Pro Gly Gly				
1010		1015	1020	
gga gat gtc aag att gaa agt cag aag ttg aac ttc aag gag aag gcc				3120
Gly Asp Val Lys Ile Glu Ser Gln Lys Leu Asn Phe Lys Glu Lys Ala				
1025		1030	1035	1040
caa gcc aaa gtg gga tcc ctt gat aac gtt ggc cac ttt cct gca gga				3168
Gln Ala Lys Val Gly Ser Leu Asp Asn Val Gly His Phe Pro Ala Gly				
1045		1050	1055	
ggt gcc gtg aag act gag ggc ggt ggc agt gag gcc ctt ccg tgt cca				3216
Gly Ala Val Lys Thr Glu Gly Gly Gly Ser Glu Ala Leu Pro Cys Pro				
1060		1065	1070	
ggc ccc ccc gct ggg gag gag cca gtc atc cct gag gct gcg cct gac				3264
Gly Pro Pro Ala Gly Glu Glu Pro Val Ile Pro Glu Ala Ala Pro Asp				
1075		1080	1085	
cgt ggc gcc cct act tca gcc agt ggc ctc agt ggc cac acc acc ctg				3312
Arg Gly Ala Pro Thr Ser Ala Ser Gly Leu Ser Ser Gly His Thr Thr Leu				
1090		1095	1100	

tca ggg ggt ggt gac caa agg gag ccc cag acc ttg gac agc cag atc 3360
 Ser Gly Gly Gly Asp Gln Arg Glu Pro Gln Thr Leu Asp Ser Gln Ile
 1105 1110 1115 1120

cag gag aca agc atc taa 3378
 Gln Glu Thr Ser Ile
 1125

<210> 152
 <211> 1125
 <212> PRT
 <213> Mus musculus

<400> 152
 Met Ala Asp Leu Ser Leu Val Asp Ala Leu Thr Glu Pro Pro Pro Glu
 1 5 10 15
 Ile Glu Gly Glu Ile Lys Arg Asp Phe Met Ala Ala Leu Glu Ala Glu
 20 25 30
 Pro Tyr Asp Asp Ile Val Gly Glu Thr Val Glu Lys Thr Glu Phe Ile
 35 40 45
 Pro Leu Leu Asp Gly Asp Glu Lys Thr Gly Asn Ser Glu Ser Lys Lys
 50 55 60
 Lys Pro Cys Leu Asp Thr Ser Gln Val Glu Gly Ile Pro Ser Ser Lys
 65 70 75 80
 Pro Thr Leu Leu Ala Asn Gly Asp His Gly Met Glu Gly Asn Asn Thr
 85 90 95
 Ala Gly Ser Pro Thr Asp Phe Leu Glu Glu Arg Val Asp Tyr Pro Asp
 100 105 110
 Tyr Gln Ser Ser Gln Asn Trp Pro Glu Asp Ala Ser Phe Cys Phe Gln
 115 120 125
 Pro Gln Gln Val Leu Asp Thr Asp Gln Ala Glu Pro Phe Asn Glu His
 130 135 140
 Arg Asp Asp Gly Leu Ala Asp Leu Leu Phe Val Ser Ser Gly Pro Thr
 145 150 155 160
 Asn Ala Ser Ala Phe Thr Glu Arg Asp Asn Pro Ser Glu Asp Ser Tyr
 165 170 175
 Gly Met Leu Pro Cys Asp Ser Phe Ala Ser Thr Ala Val Val Ser Gln
 180 185 190
 Glu Trp Ser Val Gly Ala Pro Asn Ser Pro Cys Ser Glu Ser Cys Val
 195 200 205
 Ser Pro Glu Val Thr Ile Glu Thr Leu Gln Pro Ala Thr Glu Leu Ser
 210 215 220

Lys Ala Ala Glu Val Glu Ser Val Lys Glu Gln Leu Pro Ala Lys Ala
 225 230 235 240
 Leu Glu Thr Met Ala Glu Gln Thr Thr Asp Val Val His Ser Pro Ser
 245 250 255
 Thr Asp Thr Thr Pro Gly Pro Asp Thr Glu Ala Ala Leu Ala Lys Asp
 260 265 270
 Ile Glu Glu Ile Thr Lys Pro Asp Val Ile Leu Ala Asn Val Thr Gln
 275 280 285
 Pro Ser Thr Glu Ser Asp Met Phe Leu Ala Gln Asp Met Glu Leu Leu
 290 295 300
 Thr Gly Thr Glu Ala Ala His Ala Asn Asn Ile Ile Leu Pro Thr Glu
 305 310 315 320
 Pro Asp Glu Ser Ser Thr Lys Asp Val Ala Pro Pro Met Glu Glu Glu
 325 330 335
 Ile Val Pro Gly Asn Asp Thr Thr Ser Pro Lys Glu Thr Glu Thr Thr
 340 345 350
 Leu Pro Ile Lys Met Asp Leu Ala Pro Pro Glu Asp Val Leu Leu Thr
 355 360 365
 Lys Glu Thr Glu Leu Ala Pro Ala Lys Gly Met Val Ser Leu Ser Glu
 370 375 380
 Ile Glu Glu Ala Leu Ala Lys Asn Asp Val Arg Ser Ala Glu Ile Pro
 385 390 395 400
 Val Ala Gln Glu Thr Val Val Ser Glu Thr Glu Val Val Leu Ala Thr
 405 410 415
 Glu Val Val Leu Pro Ser Asp Pro Ile Thr Thr Leu Thr Lys Asp Val
 420 425 430
 Thr Leu Pro Leu Glu Ala Glu Arg Pro Leu Val Thr Asp Met Thr Pro
 435 440 445
 Ser Leu Glu Thr Glu Met Thr Leu Gly Lys Glu Thr Ala Pro Pro Thr
 450 455 460
 Glu Thr Asn Leu Gly Met Ala Lys Asp Met Ser Pro Leu Pro Glu Ser
 465 470 475 480
 Glu Val Thr Leu Gly Lys Asp Val Val Ile Leu Pro Glu Thr Lys Val
 485 490 495
 Ala Glu Phe Asn Asn Val Thr Pro Leu Ser Glu Glu Glu Val Thr Ser
 500 505 510
 Val Lys Asp Met Ser Pro Ser Ala Glu Thr Glu Ala Pro Leu Ala Lys
 515 520 525

Asn Ala Asp Leu His Ser Gly Thr Glu Leu Ile Val Asp Asn Ser Met
 530 535 540
 Ala Pro Ala Ser Asp Leu Ala Leu Pro Leu Glu Thr Lys Val Ala Thr
 545 550 555 560
 Val Pro Ile Lys Asp Lys Gly Thr Val Gln Thr Glu Glu Lys Pro Arg
 565 570 575
 Glu Asp Ser Gln Leu Ala Ser Met Gln His Lys Gly Gln Ser Thr Val
 580 585 590
 Pro Pro Cys Thr Ala Ser Pro Glu Pro Val Lys Ala Ala Glu Gln Met
 595 600 605
 Ser Thr Leu Pro Ile Asp Ala Pro Ser Pro Leu Glu Asn Leu Glu Gln
 610 615 620
 Lys Glu Thr Pro Gly Ser Gln Pro Ser Glu Pro Cys Ser Gly Val Ser
 625 630 635 640
 Arg Gln Glu Glu Ala Lys Ala Ala Val Gly Val Thr Gly Asn Asp Ile
 645 650 655
 Thr Thr Pro Pro Asn Lys Glu Pro Pro Pro Ser Pro Glu Lys Lys Ala
 660 665 670
 Lys Pro Leu Ala Thr Thr Gln Pro Ala Lys Thr Ser Thr Ser Lys Ala
 675 680 685
 Lys Thr Gln Pro Thr Ser Leu Pro Lys Gln Pro Ala Pro Thr Thr Ser
 690 695 700
 Gly Gly Leu Asn Lys Lys Pro Met Ser Leu Ala Ser Gly Ser Val Pro
 705 710 715 720
 Ala Ala Pro His Lys Arg Pro Ala Ala Ala Thr Ala Thr Ala Arg Pro
 725 730 735
 Ser Thr Leu Pro Ala Arg Asp Val Lys Pro Lys Pro Ile Thr Glu Ala
 740 745 750
 Lys Val Ala Glu Lys Arg Thr Ser Pro Ser Lys Pro Ser Ser Ala Pro
 755 760 765
 Ala Leu Lys Pro Gly Pro Lys Thr Thr Pro Thr Val Ser Lys Ala Thr
 770 775 780
 Ser Pro Ser Thr Leu Val Ser Thr Gly Pro Ser Ser Arg Ser Pro Ala
 785 790 795 800
 Thr Thr Leu Pro Lys Arg Pro Thr Ser Ile Lys Thr Glu Gly Lys Pro
 805 810 815
 Ala Asp Val Lys Arg Met Thr Ala Lys Ser Ala Ser Ala Asp Leu Ser
 820 825 830

Arg Ser Lys Thr Thr Ser Ala Ser Ser Val Lys Arg Asn Thr Thr Pro
 835 840 845
 Thr Gly Ala Ala Pro Pro Ala Gly Met Thr Ser Thr Arg Val Lys Pro
 850 855 860
 Met Ser Ala Pro Ser Arg Ser Ser Gly Ala Leu Ser Val Asp Lys Lys
 865 870 875 880
 Pro Thr Ser Thr Lys Pro Ser Ser Ser Ala Pro Arg Val Ser Arg Leu
 885 890 895
 Ala Thr Thr Val Ser Ala Pro Asp Leu Lys Ser Val Arg Ser Lys Val
 900 905 910
 Gly Ser Thr Glu Asn Ile Lys His Gln Pro Gly Gly Gly Arg Ala Lys
 915 920 925
 Val Glu Lys Lys Thr Glu Ala Ala Thr Thr Ala Gly Lys Pro Glu Pro
 930 935 940
 Asn Ala Val Thr Lys Ala Ala Gly Ser Ile Ala Ser Ala Gln Lys Pro
 945 950 955 960
 Pro Ala Gly Lys Val Gln Ile Val Ser Lys Lys Val Ser Tyr Ser His
 965 970 975
 Ile Gln Ser Lys Cys Val Ser Lys Asp Asn Ile Lys His Val Pro Gly
 980 985 990
 Cys Gly Asn Val Gln Ile Gln Asn Lys Lys Val Asp Ile Ser Lys Val
 995 1000 1005
 Ser Ser Lys Cys Gly Ser Lys Ala Asn Ile Lys His Lys Pro Gly Gly
 1010 1015 1020
 Gly Asp Val Lys Ile Glu Ser Gln Lys Leu Asn Phe Lys Glu Lys Ala
 1025 1030 1035 1040
 Gln Ala Lys Val Gly Ser Leu Asp Asn Val Gly His Phe Pro Ala Gly
 1045 1050 1055
 Gly Ala Val Lys Thr Glu Gly Gly Gly Ser Glu Ala Leu Pro Cys Pro
 1060 1065 1070
 Gly Pro Pro Ala Gly Glu Glu Pro Val Ile Pro Glu Ala Ala Pro Asp
 1075 1080 1085
 Arg Gly Ala Pro Thr Ser Ala Ser Gly Leu Ser Gly His Thr Thr Leu
 1090 1095 1100
 Ser Gly Gly Gly Asp Gln Arg Glu Pro Gln Thr Leu Asp Ser Gln Ile
 1105 1110 1115 1120
 Gln Glu Thr Ser Ile
 1125

<210> 153
<211> 96
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 153
tcatcatccg gagctggagc cggagctggc cgatcggctg ttaaactctga aggaaagaga 60
aagtgtgacg aagttgatgg aattgatgaa gtagca 96

<210> 154
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 154
gaagaaggat ccggcacttg ggggtgtaga atgaacaccc tccaagctga gcttgacacag 60
gatttcgtgg acagtagaca tagtacttgc tacttcac 99

<210> 155
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 155
tcatcatccg gagctgga 18

<210> 156
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 156
gaagaaggat ccggcact 18

<210> 157
<211> 96
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 157
tcatcatccg gaagaaggaa acgacaaaag cgatcggctg ttaaactctga aggaaagaga 60
aagtgtgacg aagttgatgg aattgatgaa gtagca 96

<210> 158
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 158
tcatcatccg gaagaagg 18

<210> 159
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 159
tcatcatccg gaagaaggaa acgacaaaag cgatcgacaa gacttggttga aattgacaac 60

<210> 160
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 160
gaagaaggat ccggcacttg ggggtgtaga atgaacaccc tccaagctga gcttgacacag 60
gatttcgtgg acagtagaca tagtactgtt gtcaatttc 99

<210> 161
<211> 84
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 161
tcatcatccg gaagaaggaa acgacaaaag cgatcgtatc aaaaaggaa accagttgaa 60
acagacagcg aagagcaacc ttat 84

<210> 162
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 162
gaagaaggat ccggcacttg ggggtgtaga atgaacaccc tccaagctga gcttgcacag 60
gatttcgtgg acagtagaca tagtactata aggttgctc 99

<210> 163
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 163
tcatcatccg gaagaaaacg tatacgtact tacctcaagt cctgcaggcg gatgaaaaga 60

<210> 164
<211> 63
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 164
gaagaacgat cgagtaaggt gggaaggaa aggtcgagac atctcaaaac cacttctttt 60
cat 63

<210> 165
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 165
tcatcatccg gaagaaa

18

<210> 166
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide

<400> 166
gaagaacgat cgagtaag

18

<210> 167
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-1,4,5
substrate recognition sequence

<400> 167
ttagaacatg acaa

14

<210> 168
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Caspase-1,4,5
substrate recognition sequence

<400> 168
Leu Glu His Asp
1

<210> 169
<211> 1380
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-HSP27

<220>

<221> CDS

<222> (1) .. (1380)

<400> 169

atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	gtg	gtg	ccc	atc	ctg	48
Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5				10					15			
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
			35				40					45				
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55					60					
ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70				75					80		
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
			85					90						95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
		115					120					125				
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
	130					135					140					
aac	tac	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	
145					150					155					160	
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
			165					170						175		
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180					185					190			

ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu 195 200 205	624
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe 210 215 220	672
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser 225 230 235 240	720
gga ctc aga tct cga gcg gcg tcc aga gca gag tca gcc agc atg acc Gly Leu Arg Ser Arg Ala Ala Ser Arg Ala Glu Ser Ala Ser Met Thr 245 250 255	768
gag cgc cgc gtc ccc ttc tcg ctc ctg cgg ggc ccc agc tgg gac ccc Glu Arg Arg Val Pro Phe Ser Leu Leu Arg Gly Pro Ser Trp Asp Pro 260 265 270	816
ttc cgc gac tgg tac ccg cat agc cgc ctc ttc gac cag gcc ttc ggg Phe Arg Asp Trp Tyr Pro His Ser Arg Leu Phe Asp Gln Ala Phe Gly 275 280 285	864
ctg ccc cgg ctg ccg gag gag tgg tcg cag tgg tta gcc gcc agc agc Leu Pro Arg Leu Pro Glu Glu Trp Ser Gln Trp Leu Gly Gly Ser Ser 290 295 300	912
tgg cca ggc tac gtg cgc ccc ctg ccc ccc gcc gcc atc gag agc ccc Trp Pro Gly Tyr Val Arg Pro Leu Pro Pro Ala Ala Ile Glu Ser Pro 305 310 315 320	960
gca gtg gcc gcg ccc gcc tac agc cgc gcg ctc agc cgg caa ctc agc Ala Val Ala Ala Pro Ala Tyr Ser Arg Ala Leu Ser Arg Gln Leu Ser 325 330 335	1008
agc ggg gtc tcg gag atc cgg cac act gcg gac cgc tgg cgc gtg tcc Ser Gly Val Ser Glu Ile Arg His Thr Ala Asp Arg Trp Arg Val Ser 340 345 350	1056
ctg gat gtc aac cac ttc gcc ccg gac gag ctg acg gtc aag acc aag Leu Asp Val Asn His Phe Ala Pro Asp Glu Leu Thr Val Lys Thr Lys 355 360 365	1104
gat ggc gtg gtg gag atc acc ggc aag cac gag gag cgg cag gac gag Asp Gly Val Val Glu Ile Thr Gly Lys His Glu Glu Arg Gln Asp Glu 370 375 380	1152
cat ggc tac atc tcc cgg tgc ttc acg cgg aaa tac acg ctg ccc ccc His Gly Tyr Ile Ser Arg Cys Phe Thr Arg Lys Tyr Thr Leu Pro Pro 385 390 395 400	1200
ggt gtg gac ccc acc caa gtt tcc tcc tcc ctg tcc cct gag ggc aca Gly Val Asp Pro Thr Gln Val Ser Ser Ser Leu Ser Pro Glu Gly Thr 405 410 415	1248
ctg acc gtg gag gcc ccc atg ccc aag cta gcc acg cag tcc aac gag	1296

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Leu Thr Val Glu Ala Pro Met Pro Lys Leu Ala Thr Gln Ser Asn Glu
      420                      425                      430

atc acc atc cca gtc acc ttc gag tcg cgg gcc cag ctt ggg ggc cca 1344
Ile Thr Ile Pro Val Thr Phe Glu Ser Arg Ala Gln Leu Gly Gly Pro
      435                      440                      445

gaa gct gca aaa tcc gat gag act gcc gcc aag taa 1380
Glu Ala Ala Lys Ser Asp Glu Thr Ala Ala Lys
      450                      455                      460

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<210> 170
 <211> 459
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: GFP-HSP27

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<400> 170
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
  1                      5                      10                      15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
      20                      25                      30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
      35                      40                      45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
      50                      55                      60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
      65                      70                      75                      80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
      85                      90                      95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
      100                     105                     110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
      115                     120                     125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
      130                     135                     140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
      145                     150                     155                     160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
      165                     170                     175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
      180                     185                     190

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Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
 225 230 235 240
 Gly Leu Arg Ser Arg Ala Ala Ser Arg Ala Glu Ser Ala Ser Met Thr
 245 250 255
 Glu Arg Arg Val Pro Phe Ser Leu Leu Arg Gly Pro Ser Trp Asp Pro
 260 265 270
 Phe Arg Asp Trp Tyr Pro His Ser Arg Leu Phe Asp Gln Ala Phe Gly
 275 280 285
 Leu Pro Arg Leu Pro Glu Glu Trp Ser Gln Trp Leu Gly Gly Ser Ser
 290 295 300
 Trp Pro Gly Tyr Val Arg Pro Leu Pro Pro Ala Ala Ile Glu Ser Pro
 305 310 315 320
 Ala Val Ala Ala Pro Ala Tyr Ser Arg Ala Leu Ser Arg Gln Leu Ser
 325 330 335
 Ser Gly Val Ser Glu Ile Arg His Thr Ala Asp Arg Trp Arg Val Ser
 340 345 350
 Leu Asp Val Asn His Phe Ala Pro Asp Glu Leu Thr Val Lys Thr Lys
 355 360 365
 Asp Gly Val Val Glu Ile Thr Gly Lys His Glu Glu Arg Gln Asp Glu
 370 375 380
 His Gly Tyr Ile Ser Arg Cys Phe Thr Arg Lys Tyr Thr Leu Pro Pro
 385 390 395 400
 Gly Val Asp Pro Thr Gln Val Ser Ser Ser Leu Ser Pro Glu Gly Thr
 405 410 415
 Leu Thr Val Glu Ala Pro Met Pro Lys Leu Ala Thr Gln Ser Asn Glu
 420 425 430
 Ile Thr Ile Pro Val Thr Phe Glu Ser Arg Ala Gln Leu Gly Gly Pro
 435 440 445
 Glu Ala Ala Lys Ser Asp Glu Thr Ala Ala Lys
 450 455

<210> 171

<211> 2823

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-HSP70

<220>

<221> CDS

<222> (1)..(2823)

<400> 171

atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	gtg	gtg	ccc	atc	ctg	48
Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5				10					15			
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
			35				40					45				
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
		50				55					60					
ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
					70				75						80	
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	
				85					90					95		
cgc	acc	atc	ttc	ttc	aag	gac	gac	ggc	aac	tac	aag	acc	cgc	gcc	gag	336
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	
			100					105					110			
gtg	aag	ttc	gag	ggc	gac	acc	ctg	gtg	aac	cgc	atc	gag	ctg	aag	ggc	384
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	
			115				120					125				
atc	gac	ttc	aag	gag	gac	ggc	aac	atc	ctg	ggg	cac	aag	ctg	gag	tac	432
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	
			130			135					140					
aac	tac	aac	agc	cac	aac	gtc	tat	atc	atg	gcc	gac	aag	cag	aag	aac	480
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	
					150					155					160	
ggc	atc	aag	gtg	aac	ttc	aag	atc	cgc	cac	aac	atc	gag	gac	ggc	agc	528
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	
				165				170						175		
gtg	cag	ctc	gcc	gac	cac	tac	cag	cag	aac	acc	ccc	atc	ggc	gac	ggc	576
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	
			180				185						190			
ccc	gtg	ctg	ctg	ccc	gac	aac	cac	tac	ctg	agc	acc	cag	tcc	gcc	ctg	624

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195	200 205
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser	
225 230 235 240	
gga atg tcg gtg gtg ggc ata gac ctg ggc ttc cag agc tgc tac gtc	768
Gly Met Ser Val Val Gly Ile Asp Leu Gly Phe Gln Ser Cys Tyr Val	
245 250 255	
gct gtg gcc cgc gcc ggc ggc atc gag act atc gct aat gag tat agc	816
Ala Val Ala Arg Ala Gly Gly Ile Glu Thr Ile Ala Asn Glu Tyr Ser	
260 265 270	
gac cgc tgc acg ccg gct tgc att tct ttt ggt cct aag aat cgt tca	864
Asp Arg Cys Thr Pro Ala Cys Ile Ser Phe Gly Pro Lys Asn Arg Ser	
275 280 285	
att gga gca gca gct aaa agc cag gta att tct aat gca aag aac aca	912
Ile Gly Ala Ala Ala Lys Ser Gln Val Ile Ser Asn Ala Lys Asn Thr	
290 295 300	
gtc caa gga ttt aaa aga ttc cat ggc cga gca ttc tct gat cca ttt	960
Val Gln Gly Phe Lys Arg Phe His Gly Arg Ala Phe Ser Asp Pro Phe	
305 310 315 320	
gtg gag gca gaa aaa tct aac ctt gca tat gat att gtg cag tgg cct	1008
Val Glu Ala Glu Lys Ser Asn Leu Ala Tyr Asp Ile Val Gln Trp Pro	
325 330 335	
aca gga tta aca ggt ata aag gtg aca tat atg gag gaa gag cga aat	1056
Thr Gly Leu Thr Gly Ile Lys Val Thr Tyr Met Glu Glu Glu Arg Asn	
340 345 350	
ttt acc act gag caa gtg act gcc atg ctt ttg tcc aaa ctg aag gag	1104
Phe Thr Thr Glu Gln Val Thr Ala Met Leu Leu Ser Lys Leu Lys Glu	
355 360 365	
aca gcc gaa agt gtt ctt aag aag cct gta gtt gac tgt gtt gtt tcg	1152
Thr Ala Glu Ser Val Leu Lys Lys Pro Val Val Asp Cys Val Val Ser	
370 375 380	
gtt cct tgt ttc tat act gat gca gaa aga cga tca gtg atg gat gca	1200
Val Pro Cys Phe Tyr Thr Asp Ala Glu Arg Arg Ser Val Met Asp Ala	
385 390 395 400	
aca cag att gct ggt ctt aat tgc ttg cga tta atg aat gaa acc act	1248
Thr Gln Ile Ala Gly Leu Asn Cys Leu Arg Leu Met Asn Glu Thr Thr	
405 410 415	
gca gtt gct ctt gca tat gga atc tat aag cag gat ctt cct cgc tta	1296
Ala Val Ala Leu Ala Tyr Gly Ile Tyr Lys Gln Asp Leu Pro Arg Leu	

420						425						430						
gaa	gag	aaa	cca	aga	aat	gta	gtt	ttt	gta	gac	atg	ggc	cac	tct	gct	1344		
Glu	Glu	Lys	Pro	Arg	Asn	Val	Val	Phe	Val	Asp	Met	Gly	His	Ser	Ala			
		435					440					445						
tat	caa	gtt	tct	gta	tgt	gca	ttt	aat	aga	gga	aaa	ctg	aaa	gtt	ctg	1392		
Tyr	Gln	Val	Ser	Val	Cys	Ala	Phe	Asn	Arg	Gly	Lys	Leu	Lys	Val	Leu			
	450					455					460							
gcc	act	gca	ttt	gac	acg	aca	ttg	gga	ggg	aga	aaa	ttt	gat	gaa	gtg	1440		
Ala	Thr	Ala	Phe	Asp	Thr	Thr	Leu	Gly	Gly	Arg	Lys	Phe	Asp	Glu	Val			
465					470					475					480			
tta	gta	aat	cac	ttc	tgt	gaa	gaa	ttt	ggg	aag	aaa	tac	aag	cta	gac	1488		
Leu	Val	Asn	His	Phe	Cys	Glu	Glu	Phe	Gly	Lys	Lys	Tyr	Lys	Leu	Asp			
				485					490					495				
att	aag	tcc	aaa	atc	cgt	gca	tta	tta	cga	ctc	tct	cag	gag	tgt	gag	1536		
Ile	Lys	Ser	Lys	Ile	Arg	Ala	Leu	Leu	Arg	Leu	Ser	Gln	Glu	Cys	Glu			
			500					505					510					
aaa	ctc	aag	aaa	ttg	atg	agt	gca	aat	gct	tca	gat	ctc	cct	ttg	agc	1584		
Lys	Leu	Lys	Lys	Leu	Met	Ser	Ala	Asn	Ala	Ser	Asp	Leu	Pro	Leu	Ser			
		515					520					525						
att	gaa	tgt	ttt	atg	aat	gat	gtt	gat	gta	tct	gga	act	atg	aat	aga	1632		
Ile	Glu	Cys	Phe	Met	Asn	Asp	Val	Asp	Val	Ser	Gly	Thr	Met	Asn	Arg			
	530					535					540							
ggc	aaa	ttt	ctg	gag	atg	tgc	aat	gat	ctc	tta	gct	aga	gtg	gag	cca	1680		
Gly	Lys	Phe	Leu	Glu	Met	Cys	Asn	Asp	Leu	Leu	Ala	Arg	Val	Glu	Pro			
545					550					555					560			
cca	ctt	cgt	agt	gtt	ttg	gaa	caa	acc	aag	tta	aag	aaa	gaa	gat	att	1728		
Pro	Leu	Arg	Ser	Val	Leu	Glu	Gln	Thr	Lys	Leu	Lys	Lys	Glu	Asp	Ile			
				565					570					575				
tat	gca	gtg	gag	ata	gtt	ggg	ggg	gct	aca	cga	atc	cct	gcg	gta	aaa	1776		
Tyr	Ala	Val	Glu	Ile	Val	Gly	Gly	Ala	Thr	Arg	Ile	Pro	Ala	Val	Lys			
			580					585					590					
gag	aag	atc	agc	aaa	ttt	ttc	ggg	aaa	gaa	ctt	agt	aca	aca	tta	aat	1824		
Glu	Lys	Ile	Ser	Lys	Phe	Phe	Gly	Lys	Glu	Leu	Ser	Thr	Thr	Leu	Asn			
		595					600					605						
gct	gat	gaa	gct	gtc	act	cga	ggc	tgt	gca	ttg	cag	tgt	gcc	atc	tta	1872		
Ala	Asp	Glu	Ala	Val	Thr	Arg	Gly	Cys	Ala	Leu	Gln	Cys	Ala	Ile	Leu			
	610					615					620							
tcg	cct	gct	ttc	aaa	gtc	aga	gaa	ttt	tct	atc	act	gat	gta	gta	cca	1920		
Ser	Pro	Ala	Phe	Lys	Val	Arg	Glu	Phe	Ser	Ile	Thr	Asp	Val	Val	Pro			
625					630					635					640			
tat	cca	ata	tct	ctg	aga	tgg	aat	tct	cca	gct	gaa	gaa	ggg	tca	agt	1968		
Tyr	Pro	Ile	Ser	Leu	Arg	Trp	Asn	Ser	Pro	Ala	Glu	Glu	Gly	Ser	Ser			
				645					650				655					

gac tgt gaa gtc ttt tcc aaa aat cat gct gct cct ttc tct aaa gtt	2016
Asp Cys Glu Val Phe Ser Lys Asn His Ala Ala Pro Phe Ser Lys Val	
660 665 670	
ctt aca ttt tat aga aag gaa cct ttc act ctt gag gcc tac tac agc	2064
Leu Thr Phe Tyr Arg Lys Glu Pro Phe Thr Leu Glu Ala Tyr Tyr Ser	
675 680 685	
tct cct cag gat ttg ccc tat cca gat cct gct ata gct cag ttt tca	2112
Ser Pro Gln Asp Leu Pro Tyr Pro Asp Pro Ala Ile Ala Gln Phe Ser	
690 695 700	
gtt cag aaa gtc act cct cag tct gat ggc tcc agt tca aaa gtg aaa	2160
Val Gln Lys Val Thr Pro Gln Ser Asp Gly Ser Ser Ser Lys Val Lys	
705 710 715 720	
gtc aaa gtt cga gta aat gtc cat ggc att ttc agt gtg tcc agt gca	2208
Val Lys Val Arg Val Asn Val His Gly Ile Phe Ser Val Ser Ser Ala	
725 730 735	
tct tta gtg gag gtt cac aag tct gag gaa aat gag gag cca atg gaa	2256
Ser Leu Val Glu Val His Lys Ser Glu Glu Asn Glu Glu Pro Met Glu	
740 745 750	
aca gat cag aat gca aag gag gaa gag aag atg caa gtg gac cag gag	2304
Thr Asp Gln Asn Ala Lys Glu Glu Glu Lys Met Gln Val Asp Gln Glu	
755 760 765	
gaa cca cat gtt gaa gag caa cag cag cag aca cca gca gaa aat aag	2352
Glu Pro His Val Glu Glu Gln Gln Gln Gln Thr Pro Ala Glu Asn Lys	
770 775 780	
gca gag tct gaa gaa atg gag acc tct caa gct gga tcc aag gat aaa	2400
Ala Glu Ser Glu Glu Met Glu Thr Ser Gln Ala Gly Ser Lys Asp Lys	
785 790 795 800	
aag atg gac caa cca ccc caa tgc caa gaa ggc aaa agt gaa gac cag	2448
Lys Met Asp Gln Pro Pro Gln Cys Gln Glu Gly Lys Ser Glu Asp Gln	
805 810 815	
tac tgt gga cct gcc aat cga gaa tca gct ata tgg cag ata gac aga	2496
Tyr Cys Gly Pro Ala Asn Arg Glu Ser Ala Ile Trp Gln Ile Asp Arg	
820 825 830	
gag atg ctc aac ttg tac att gaa aat gag ggt aag atg atc atg cag	2544
Glu Met Leu Asn Leu Tyr Ile Glu Asn Glu Gly Lys Met Ile Met Gln	
835 840 845	
gat aaa ctg gag aag gag cgg aat gat gct aag aac gca gtg gag gaa	2592
Asp Lys Leu Glu Lys Glu Arg Asn Asp Ala Lys Asn Ala Val Glu Glu	
850 855 860	
tat gtg tat gaa atg aga gac aag ctt agt ggt gaa tat gag aag ttt	2640
Tyr Val Tyr Glu Met Arg Asp Lys Leu Ser Gly Glu Tyr Glu Lys Phe	
865 870 875 880	

gtg agt gaa gat gat cgt aac agt ttt act ttg aaa ctg gaa gat act 2688
Val Ser Glu Asp Asp Arg Asn Ser Phe Thr Leu Lys Leu Glu Asp Thr
885 890 895

gaa aat tgg ttg tat gag gat gga gaa gac cag cca aag caa gtt tat 2736
Glu Asn Trp Leu Tyr Glu Asp Gly Glu Asp Gln Pro Lys Gln Val Tyr
900 905 910

ggt gat aag ttg gct gaa tta aaa aat cta ggt caa cct att aag ata 2784
Val Asp Lys Leu Ala Glu Leu Lys Asn Leu Gly Gln Pro Ile Lys Ile
915 920 925

cgt ttc cag gaa tct gaa gaa cga cca aat tat ttg aag 2823
Arg Phe Gln Glu Ser Glu Glu Arg Pro Asn Tyr Leu Lys
930 935 940

<210> 172

<211> 941

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-HSP70

<400> 172

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Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
50 55 60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser

165										170					175				
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly				
			180						185					190					
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu				
		195					200					205							
Ser	Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe				
	210					215					220								
Val	Thr	Ala	Ala	Gly	Ile	Thr	Leu	Gly	Met	Asp	Glu	Leu	Tyr	Lys	Ser				
225					230					235					240				
Gly	Met	Ser	Val	Val	Gly	Ile	Asp	Leu	Gly	Phe	Gln	Ser	Cys	Tyr	Val				
				245					250					255					
Ala	Val	Ala	Arg	Ala	Gly	Gly	Ile	Glu	Thr	Ile	Ala	Asn	Glu	Tyr	Ser				
			260					265					270						
Asp	Arg	Cys	Thr	Pro	Ala	Cys	Ile	Ser	Phe	Gly	Pro	Lys	Asn	Arg	Ser				
		275					280						285						
Ile	Gly	Ala	Ala	Ala	Lys	Ser	Gln	Val	Ile	Ser	Asn	Ala	Lys	Asn	Thr				
	290					295					300								
Val	Gln	Gly	Phe	Lys	Arg	Phe	His	Gly	Arg	Ala	Phe	Ser	Asp	Pro	Phe				
305					310					315					320				
Val	Glu	Ala	Glu	Lys	Ser	Asn	Leu	Ala	Tyr	Asp	Ile	Val	Gln	Trp	Pro				
				325					330					335					
Thr	Gly	Leu	Thr	Gly	Ile	Lys	Val	Thr	Tyr	Met	Glu	Glu	Glu	Arg	Asn				
			340					345						350					
Phe	Thr	Thr	Glu	Gln	Val	Thr	Ala	Met	Leu	Leu	Ser	Lys	Leu	Lys	Glu				
			355				360						365						
Thr	Ala	Glu	Ser	Val	Leu	Lys	Lys	Pro	Val	Val	Asp	Cys	Val	Val	Ser				
	370					375					380								
Val	Pro	Cys	Phe	Tyr	Thr	Asp	Ala	Glu	Arg	Arg	Ser	Val	Met	Asp	Ala				
385					390					395					400				
Thr	Gln	Ile	Ala	Gly	Leu	Asn	Cys	Leu	Arg	Leu	Met	Asn	Glu	Thr	Thr				
				405					410					415					
Ala	Val	Ala	Leu	Ala	Tyr	Gly	Ile	Tyr	Lys	Gln	Asp	Leu	Pro	Arg	Leu				
			420					425					430						
Glu	Glu	Lys	Pro	Arg	Asn	Val	Val	Phe	Val	Asp	Met	Gly	His	Ser	Ala				
		435					440					445							
Tyr	Gln	Val	Ser	Val	Cys	Ala	Phe	Asn	Arg	Gly	Lys	Leu	Lys	Val	Leu				
	450					455					460								
Ala	Thr	Ala	Phe	Asp	Thr	Thr	Leu	Gly	Gly	Arg	Lys	Phe	Asp	Glu	Val				

465		470		475		480
Leu Val Asn His Phe Cys Glu Glu Phe Gly Lys Lys Tyr Lys Leu Asp						
	485			490		495
Ile Lys Ser Lys Ile Arg Ala Leu Leu Arg Leu Ser Gln Glu Cys Glu						
	500			505		510
Lys Leu Lys Lys Leu Met Ser Ala Asn Ala Ser Asp Leu Pro Leu Ser						
	515			520		525
Ile Glu Cys Phe Met Asn Asp Val Asp Val Ser Gly Thr Met Asn Arg						
	530			535		540
Gly Lys Phe Leu Glu Met Cys Asn Asp Leu Leu Ala Arg Val Glu Pro						
	545			550		555
Pro Leu Arg Ser Val Leu Glu Gln Thr Lys Leu Lys Lys Glu Asp Ile						
	565			570		575
Tyr Ala Val Glu Ile Val Gly Gly Ala Thr Arg Ile Pro Ala Val Lys						
	580			585		590
Glu Lys Ile Ser Lys Phe Phe Gly Lys Glu Leu Ser Thr Thr Leu Asn						
	595			600		605
Ala Asp Glu Ala Val Thr Arg Gly Cys Ala Leu Gln Cys Ala Ile Leu						
	610			615		620
Ser Pro Ala Phe Lys Val Arg Glu Phe Ser Ile Thr Asp Val Val Pro						
	625			630		635
Tyr Pro Ile Ser Leu Arg Trp Asn Ser Pro Ala Glu Glu Gly Ser Ser						
	645			650		655
Asp Cys Glu Val Phe Ser Lys Asn His Ala Ala Pro Phe Ser Lys Val						
	660			665		670
Leu Thr Phe Tyr Arg Lys Glu Pro Phe Thr Leu Glu Ala Tyr Tyr Ser						
	675			680		685
Ser Pro Gln Asp Leu Pro Tyr Pro Asp Pro Ala Ile Ala Gln Phe Ser						
	690			695		700
Val Gln Lys Val Thr Pro Gln Ser Asp Gly Ser Ser Ser Lys Val Lys						
	705			710		715
Val Lys Val Arg Val Asn Val His Gly Ile Phe Ser Val Ser Ser Ala						
	725			730		735
Ser Leu Val Glu Val His Lys Ser Glu Glu Asn Glu Glu Pro Met Glu						
	740			745		750
Thr Asp Gln Asn Ala Lys Glu Glu Glu Lys Met Gln Val Asp Gln Glu						
	755			760		765
Glu Pro His Val Glu Glu Gln Gln Gln Gln Thr Pro Ala Glu Asn Lys						

770 775 780

Ala Glu Ser Glu Glu Met Glu Thr Ser Gln Ala Gly Ser Lys Asp Lys
 785 790 795 800

Lys Met Asp Gln Pro Pro Gln Cys Gln Glu Gly Lys Ser Glu Asp Gln
 805 810 815

Tyr Cys Gly Pro Ala Asn Arg Glu Ser Ala Ile Trp Gln Ile Asp Arg
 820 825 830

Glu Met Leu Asn Leu Tyr Ile Glu Asn Glu Gly Lys Met Ile Met Gln
 835 840 845

Asp Lys Leu Glu Lys Glu Arg Asn Asp Ala Lys Asn Ala Val Glu Glu
 850 855 860

Tyr Val Tyr Glu Met Arg Asp Lys Leu Ser Gly Glu Tyr Glu Lys Phe
 865 870 875 880

Val Ser Glu Asp Asp Arg Asn Ser Phe Thr Leu Lys Leu Glu Asp Thr
 885 890 895

Glu Asn Trp Leu Tyr Glu Asp Gly Glu Asp Gln Pro Lys Gln Val Tyr
 900 905 910

Val Asp Lys Leu Ala Glu Leu Lys Asn Leu Gly Gln Pro Ile Lys Ile
 915 920 925

Arg Phe Gln Glu Ser Glu Glu Arg Pro Asn Tyr Leu Lys
 930 935 940

<210> 173
 <211> 2674
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: GFP-HSC70

<220>
 <221> CDS
 <222> (1)..(2673)

<400> 173

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Met	Val	Ser	Lys	Gly	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu		
1				5				10					15			

gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			

gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
		35					40					45				

tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc	192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr	
50 55 60	
ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag	240
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys	
65 70 75 80	
cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag	288
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu	
85 90 95	
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag	336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu	
100 105 110	
gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc	384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly	
115 120 125	
atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac	432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr	
130 135 140	
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac	480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn	
145 150 155 160	
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc	528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser	
165 170 175	
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc	576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly	
180 185 190	
ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg	624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu	
195 200 205	
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser	
225 230 235 240	
gga ctc aga tct atg tcc aag gga cct gca gtt ggt att gat ctt ggc	768
Gly Leu Arg Ser Met Ser Lys Gly Pro Ala Val Gly Ile Asp Leu Gly	
245 250 255	
acc acc tac tct tgt gtg ggt gtt ttc cag cac gga aaa gtc gag ata	816
Thr Thr Tyr Ser Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile	
260 265 270	

att gcc aat gat cag gga aac cga acc act cca agc tat gtc gcc ttt Ile Ala Asn Asp Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe 275 280 285	864
acg gac act gaa cgg ttg atc ggt gat gcc gca aag aat caa gtt gca Thr Asp Thr Glu Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala 290 295 300	912
atg aac ccc acc aac aca gtt ttt gat gcc aaa cgt ctg att gga cgc Met Asn Pro Thr Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg 305 310 315 320	960
aga ttt gat gat gct gtt gtc cag tct gat atg aaa cat tgg ccc ttt Arg Phe Asp Asp Ala Val Val Gln Ser Asp Met Lys His Trp Pro Phe 325 330 335	1008
atg gtg gtg aat gat gct ggc agg ccc aag gtc caa gta gaa tac aag Met Val Val Asn Asp Ala Gly Arg Pro Lys Val Gln Val Glu Tyr Lys 340 345 350	1056
gga gag acc aaa agc ttc tat cca gag gag gtg tct tct atg gtt ctg Gly Glu Thr Lys Ser Phe Tyr Pro Glu Glu Val Ser Ser Met Val Leu 355 360 365	1104
aca aag atg aag gaa att gca gaa gcc tac ctt ggg aag act gtt acc Thr Lys Met Lys Glu Ile Ala Glu Ala Tyr Leu Gly Lys Thr Val Thr 370 375 380	1152
aat gct gtg gtc aca gtg cca gct tac ttt aat gac tct cag cgt cag Asn Ala Val Val Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln 385 390 395 400	1200
gct acc aaa gat gct gga act att gct ggt ctc aat gta ctt aga att Ala Thr Lys Asp Ala Gly Thr Ile Ala Gly Leu Asn Val Leu Arg Ile 405 410 415	1248
att aat gag cca act gct gct gct att gct tac ggc tta gac aaa aag Ile Asn Glu Pro Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Lys Lys 420 425 430	1296
gtt gga gca gaa aga aac gtg ctc atc ttt gac ctg gga ggt ggc act Val Gly Ala Glu Arg Asn Val Leu Ile Phe Asp Leu Gly Gly Thr 435 440 445	1344
ttt gat gtg tca atc ctc act att gag gat gga atc ttt gag gtc aag Phe Asp Val Ser Ile Leu Thr Ile Glu Asp Gly Ile Phe Glu Val Lys 450 455 460	1392
tct aca gct gga gac acc cac ttg ggt gga gaa gat ttt gac aac cga Ser Thr Ala Gly Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg 465 470 475 480	1440
atg gtc aac cat ttt att gct gag ttt aag cgc aag cat aag aag gac Met Val Asn His Phe Ile Ala Glu Phe Lys Arg Lys His Lys Lys Asp 485 490 495	1488
atc agt gag aac aag aga gct gta aga cgc ctc cgt act gct tgt gaa	1536

Ile Ser Glu Asn Lys Arg Ala Val Arg Arg Leu Arg Thr Ala Cys Glu	
500 505 510	
cgt gct aag cgt acc ctc tct tcc agc acc cag gcc agt att gag atc	1584
Arg Ala Lys Arg Thr Leu Ser Ser Ser Thr Gln Ala Ser Ile Glu Ile	
515 520 525	
gat tct ctc tat gaa gga atc gac ttc tat acc tcc att acc cgt gcc	1632
Asp Ser Leu Tyr Glu Gly Ile Asp Phe Tyr Thr Ser Ile Thr Arg Ala	
530 535 540	
cga ttt gaa gaa ctg aat gct gac ctg ttc cgt ggc acc ctg gac cca	1680
Arg Phe Glu Glu Leu Asn Ala Asp Leu Phe Arg Gly Thr Leu Asp Pro	
545 550 555 560	
gta gag aaa gcc ctt cga gat gcc aaa cta gac aag tca cag att cat	1728
Val Glu Lys Ala Leu Arg Asp Ala Lys Leu Asp Lys Ser Gln Ile His	
565 570 575	
gat att gtc ctg gtt ggt ggt tct act cgt atc ccc aag att cag aag	1776
Asp Ile Val Leu Val Gly Gly Ser Thr Arg Ile Pro Lys Ile Gln Lys	
580 585 590	
ctt ctc caa gac ttc ttc aat gga aaa gaa ctg aat aag agc atc aac	1824
Leu Leu Gln Asp Phe Phe Asn Gly Lys Glu Leu Asn Lys Ser Ile Asn	
595 600 605	
cct gat gaa gct gtt gct tat ggt gca gct gtc cag gca gcc atc ttg	1872
Pro Asp Glu Ala Val Ala Tyr Gly Ala Ala Val Gln Ala Ala Ile Leu	
610 615 620	
tct gga gac aag tct gag aat gtt caa gat ttg ctg ctc ttg gat gtc	1920
Ser Gly Asp Lys Ser Glu Asn Val Gln Asp Leu Leu Leu Asp Val	
625 630 635 640	
act cct ctt tcc ctt ggt att gaa act gct ggt gga gtc atg act gtc	1968
Thr Pro Leu Ser Leu Gly Ile Glu Thr Ala Gly Gly Val Met Thr Val	
645 650 655	
ctc atc aag cgt aat acc acc att cct acc aag cag aca cag acc ttc	2016
Leu Ile Lys Arg Asn Thr Thr Ile Pro Thr Lys Gln Thr Gln Thr Phe	
660 665 670	
act acc tat tct gac aac cag cct ggt gtg ctt att cag gtt tat gaa	2064
Thr Thr Tyr Ser Asp Asn Gln Pro Gly Val Leu Ile Gln Val Tyr Glu	
675 680 685	
ggc gag cgt gcc atg aca aag gat aac aac ctg ctt ggc aag ttt gaa	2112
Gly Glu Arg Ala Met Thr Lys Asp Asn Asn Leu Leu Gly Lys Phe Glu	
690 695 700	
ctc aca ggc ata cct cct gca ccc cga ggt gtt cct cag att gaa gtc	2160
Leu Thr Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln Ile Glu Val	
705 710 715 720	
act ttt gac att gat gcc aat ggt ata ctc aat gtc tct gct gtg gac	2208
Thr Phe Asp Ile Asp Ala Asn Gly Ile Leu Asn Val Ser Ala Val Asp	

	725	730	735	
aag agt acg gga aaa gag aac aag att act atc act aat gac aag ggc				2256
Lys Ser Thr Gly Lys Glu Asn Lys Ile Thr Ile Thr Asn Asp Lys Gly	740	745	750	
cgt ttg agc aag gaa gac att gaa cgt atg gtc cag gaa gct gag aag				2304
Arg Leu Ser Lys Glu Asp Ile Glu Arg Met Val Gln Glu Ala Glu Lys	755	760	765	
tac aaa gct gaa gat gag aag cag agg gac aag gtg tca tcc aag aat				2352
Tyr Lys Ala Glu Asp Glu Lys Gln Arg Asp Lys Val Ser Ser Lys Asn	770	775	780	
tca ctt gag tcc tat gcc ttc aac atg aaa gca act gtt gaa gat gag				2400
Ser Leu Glu Ser Tyr Ala Phe Asn Met Lys Ala Thr Val Glu Asp Glu	785	790	795	800
aaa ctt caa ggc aag att aac gat gag gac aaa cag aag att ctg gac				2448
Lys Leu Gln Gly Lys Ile Asn Asp Glu Asp Lys Lys Gln Lys Ile Leu Asp	805	810	815	
aag tgt aat gaa att atc aac tgg ctt gat aag aat cag act gct gag				2496
Lys Cys Asn Glu Ile Ile Asn Trp Leu Asp Lys Asn Gln Thr Ala Glu	820	825	830	
aag gaa gaa ttt gaa cat caa cag aaa gag ctg gag aaa gtt tgc aac				2544
Lys Glu Glu Phe Glu His Gln Gln Lys Glu Leu Glu Lys Val Cys Asn	835	840	845	
ccc atc atc acc aag ctg tac cag agt gca gga ggc atg cca gga gga				2592
Pro Ile Ile Thr Lys Leu Tyr Gln Ser Ala Gly Gly Met Pro Gly Gly	850	855	860	
atg cct ggg gga ttt cct ggt ggt gga gct cct ccc tct ggt ggt gct				2640
Met Pro Gly Gly Phe Pro Gly Gly Gly Ala Pro Pro Ser Gly Gly Ala	865	870	875	880
tcc tca ggg ccc acc att gaa gag gtt gat taa g				2674
Ser Ser Gly Pro Thr Ile Glu Glu Val Asp	885	890		

<210> 174

<211> 890

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-HSC70

<400> 174

Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu
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Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly
	20				25							30			

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60
 Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
 165 170 175
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
 225 230 235 240
 Gly Leu Arg Ser Met Ser Lys Gly Pro Ala Val Gly Ile Asp Leu Gly
 245 250 255
 Thr Thr Tyr Ser Cys Val Gly Val Phe Gln His Gly Lys Val Glu Ile
 260 265 270
 Ile Ala Asn Asp Gln Gly Asn Arg Thr Thr Pro Ser Tyr Val Ala Phe
 275 280 285
 Thr Asp Thr Glu Arg Leu Ile Gly Asp Ala Ala Lys Asn Gln Val Ala
 290 295 300
 Met Asn Pro Thr Asn Thr Val Phe Asp Ala Lys Arg Leu Ile Gly Arg
 305 310 315 320
 Arg Phe Asp Asp Ala Val Val Gln Ser Asp Met Lys His Trp Pro Phe
 325 330 335

Met Val Val Asn Asp Ala Gly Arg Pro Lys Val Gln Val Glu Tyr Lys
 340 345 350
 Gly Glu Thr Lys Ser Phe Tyr Pro Glu Glu Val Ser Ser Met Val Leu
 355 360 365
 Thr Lys Met Lys Glu Ile Ala Glu Ala Tyr Leu Gly Lys Thr Val Thr
 370 375 380
 Asn Ala Val Val Thr Val Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln
 385 390 395 400
 Ala Thr Lys Asp Ala Gly Thr Ile Ala Gly Leu Asn Val Leu Arg Ile
 405 410 415
 Ile Asn Glu Pro Thr Ala Ala Ala Ile Ala Tyr Gly Leu Asp Lys Lys
 420 425 430
 Val Gly Ala Glu Arg Asn Val Leu Ile Phe Asp Leu Gly Gly Gly Thr
 435 440 445
 Phe Asp Val Ser Ile Leu Thr Ile Glu Asp Gly Ile Phe Glu Val Lys
 450 455 460
 Ser Thr Ala Gly Asp Thr His Leu Gly Gly Glu Asp Phe Asp Asn Arg
 465 470 475 480
 Met Val Asn His Phe Ile Ala Glu Phe Lys Arg Lys His Lys Lys Asp
 485 490 495
 Ile Ser Glu Asn Lys Arg Ala Val Arg Arg Leu Arg Thr Ala Cys Glu
 500 505 510
 Arg Ala Lys Arg Thr Leu Ser Ser Ser Thr Gln Ala Ser Ile Glu Ile
 515 520 525
 Asp Ser Leu Tyr Glu Gly Ile Asp Phe Tyr Thr Ser Ile Thr Arg Ala
 530 535 540
 Arg Phe Glu Glu Leu Asn Ala Asp Leu Phe Arg Gly Thr Leu Asp Pro
 545 550 555 560
 Val Glu Lys Ala Leu Arg Asp Ala Lys Leu Asp Lys Ser Gln Ile His
 565 570 575
 Asp Ile Val Leu Val Gly Gly Ser Thr Arg Ile Pro Lys Ile Gln Lys
 580 585 590
 Leu Leu Gln Asp Phe Phe Asn Gly Lys Glu Leu Asn Lys Ser Ile Asn
 595 600 605
 Pro Asp Glu Ala Val Ala Tyr Gly Ala Ala Val Gln Ala Ala Ile Leu
 610 615 620
 Ser Gly Asp Lys Ser Glu Asn Val Gln Asp Leu Leu Leu Leu Asp Val
 625 630 635 640

Thr Pro Leu Ser Leu Gly Ile Glu Thr Ala Gly Gly Val Met Thr Val
 645 650 655
 Leu Ile Lys Arg Asn Thr Thr Ile Pro Thr Lys Gln Thr Gln Thr Phe
 660 665 670
 Thr Thr Tyr Ser Asp Asn Gln Pro Gly Val Leu Ile Gln Val Tyr Glu
 675 680 685
 Gly Glu Arg Ala Met Thr Lys Asp Asn Asn Leu Leu Gly Lys Phe Glu
 690 695 700
 Leu Thr Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln Ile Glu Val
 705 710 715 720
 Thr Phe Asp Ile Asp Ala Asn Gly Ile Leu Asn Val Ser Ala Val Asp
 725 730 735
 Lys Ser Thr Gly Lys Glu Asn Lys Ile Thr Ile Thr Asn Asp Lys Gly
 740 745 750
 Arg Leu Ser Lys Glu Asp Ile Glu Arg Met Val Gln Glu Ala Glu Lys
 755 760 765
 Tyr Lys Ala Glu Asp Glu Lys Gln Arg Asp Lys Val Ser Ser Lys Asn
 770 775 780
 Ser Leu Glu Ser Tyr Ala Phe Asn Met Lys Ala Thr Val Glu Asp Glu
 785 790 795 800
 Lys Leu Gln Gly Lys Ile Asn Asp Glu Asp Lys Gln Lys Ile Leu Asp
 805 810 815
 Lys Cys Asn Glu Ile Ile Asn Trp Leu Asp Lys Asn Gln Thr Ala Glu
 820 825 830
 Lys Glu Glu Phe Glu His Gln Gln Lys Glu Leu Glu Lys Val Cys Asn
 835 840 845
 Pro Ile Ile Thr Lys Leu Tyr Gln Ser Ala Gly Gly Met Pro Gly Gly
 850 855 860
 Met Pro Gly Gly Phe Pro Gly Gly Gly Ala Pro Pro Ser Gly Gly Ala
 865 870 875 880
 Ser Ser Gly Pro Thr Ile Glu Glu Val Asp
 885 890

<210> 175

<211> 2458

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-HSF1

<220>

<221> CDS

<222> (1)..(2349)

<400> 175

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atg gtg agc aag ggc gag gag ctg ttc acc ggg gtg gtg ccc atc ctg      48
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
  1             5             10             15

gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc ggc      96
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
          20             25             30

gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc     144
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
          35             40             45

tgc acc acc ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc     192
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
          50             55             60

ctg acc tac ggc gtg cag tgc ttc agc cgc tac ccc gac cac atg aag     240
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
          65             70             75             80

cag cac gac ttc ttc aag tcc gcc atg ccc gaa ggc tac gtc cag gag     288
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
          85             90             95

cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag     336
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
          100            105            110

gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc     384
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
          115            120            125

atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac     432
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
          130            135            140

aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac     480
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
          145            150            155            160

ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc     528
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
          165            170            175

gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc     576
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
          180            185            190

ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg     624
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
          195            200            205

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agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe	
210 215 220	
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc	720
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser	
225 230 235 240	
gga ctc aga tct cga gct caa gct tcg aat tct gca gtc gag atg gat	768
Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Ala Val Glu Met Asp	
245 250 255	
ctg ccc gtg ggc ccc ggc gcg gcg ggg ccc agc aac gtc ccg gcc ttc	816
Leu Pro Val Gly Pro Gly Ala Ala Gly Pro Ser Asn Val Pro Ala Phe	
260 265 270	
ctg acc aag ctg tgg acc ctc gtg agc gac ccg gac acc gac gcg ctc	864
Leu Thr Lys Leu Trp Thr Leu Val Ser Asp Pro Asp Thr Asp Ala Leu	
275 280 285	
atc tgc tgg agc ccg agc ggg aac agc ttc cac gtg ttc gac cag ggc	912
Ile Cys Trp Ser Pro Ser Gly Asn Ser Phe His Val Phe Asp Gln Gly	
290 295 300	
cag ttt gcc aag gag gtg ctg ccc aag tac ttc aag cac aac aac atg	960
Gln Phe Ala Lys Glu Val Leu Pro Lys Tyr Phe Lys His Asn Asn Met	
305 310 315 320	
gcc agc ttc gtg cgg cag ctc aac atg tat ggc ttc cgg aaa gtg gtc	1008
Ala Ser Phe Val Arg Gln Leu Asn Met Tyr Gly Phe Arg Lys Val Val	
325 330 335	
cac atc gag cag ggc ggc ctg gtc aag cca gag aga gac gac acg gag	1056
His Ile Glu Gln Gly Gly Leu Val Lys Pro Glu Arg Asp Asp Thr Glu	
340 345 350	
ttc cag cac cca tgc ttc ctg cgt ggc cag gag cag ctc ctt gag aac	1104
Phe Gln His Pro Cys Phe Leu Arg Gly Gln Glu Gln Leu Leu Glu Asn	
355 360 365	
atc aag agg aaa gtg acc agt gtg tcc acc ctg aag agt gaa gac ata	1152
Ile Lys Arg Lys Val Thr Ser Val Ser Thr Leu Lys Ser Glu Asp Ile	
370 375 380	
aag atc cgc cag gac agc gtc acc aag ctg ctg acg gac gtg cag ctg	1200
Lys Ile Arg Gln Asp Ser Val Thr Lys Leu Leu Thr Asp Val Gln Leu	
385 390 395 400	
atg aag ggg aag cag gag tgc atg gac tcc aag ctc ctg gcc atg aag	1248
Met Lys Gly Lys Gln Glu Cys Met Asp Ser Lys Leu Leu Ala Met Lys	
405 410 415	
cat gag aat gag gct ctg tgg cgg gag gtg gcc agc ctt cgg cag aag	1296
His Glu Asn Glu Ala Leu Trp Arg Glu Val Ala Ser Leu Arg Gln Lys	
420 425 430	

cat gcc cag caa cag aaa gtc gtc aac aag ctc att cag ttc ctg atc His Ala Gln Gln Lys Val Val Asn Lys Leu Ile Gln Phe Leu Ile 435 440 445	1344
tca ctg gtg cag tca aac cgg atc ctg ggg gtg aag aga aag atc ccc Ser Leu Val Gln Ser Asn Arg Ile Leu Gly Val Lys Arg Lys Ile Pro 450 455 460	1392
ctg atg ctg aac gac agt ggc tca gca cat tcc atg ccc aag tat agc Leu Met Leu Asn Asp Ser Gly Ser Ala His Ser Met Pro Lys Tyr Ser 465 470 475 480	1440
cgg cag ttc tcc ctg gag cac gtc cac ggc tgg ggc ccc tac tgg gcc Arg Gln Phe Ser Leu Glu His Val His Gly Ser Gly Pro Tyr Ser Ala 485 490 495	1488
ccc tcc cca gcc tac agc agc tcc agc ctc tac gcc cct gat gct gtg Pro Ser Pro Ala Tyr Ser Ser Ser Ser Leu Tyr Ala Pro Asp Ala Val 500 505 510	1536
gcc agc tct gga ccc atc atc tcc gac atc acc gag ctg gct cct gcc Ala Ser Ser Gly Pro Ile Ile Ser Asp Ile Thr Glu Leu Ala Pro Ala 515 520 525	1584
agc ccc atg gcc tcc ccc ggc ggg agc ata gac gag agg ccc cta tcc Ser Pro Met Ala Ser Pro Gly Gly Ser Ile Asp Glu Arg Pro Leu Ser 530 535 540	1632
agc agc ccc ctg gtg cgt gtc aag gag gag ccc ccc agc cgg cct cag Ser Ser Pro Leu Val Arg Val Lys Glu Glu Pro Pro Ser Pro Pro Gln 545 550 555 560	1680
agc ccc cgg gta gag gag gcg agt ccc ggg cgc cca tct tcc gtg gac Ser Pro Arg Val Glu Glu Ala Ser Pro Gly Arg Pro Ser Ser Val Asp 565 570 575	1728
acc ctc ttg tcc ccg acc gcc ctc att gac tcc atc ctg cgg gag agt Thr Leu Leu Ser Pro Thr Ala Leu Ile Asp Ser Ile Leu Arg Glu Ser 580 585 590	1776
gaa cct gcc ccc gcc tcc gtc aca gcc ctc acg gac gcc agg ggc cac Glu Pro Ala Pro Ala Ser Val Thr Ala Leu Thr Asp Ala Arg Gly His 595 600 605	1824
acg gac acc gag ggc cgg cct ccc tcc ccc cgg ccc acc tcc acc cct Thr Asp Thr Glu Gly Arg Pro Pro Ser Pro Pro Pro Thr Ser Thr Pro 610 615 620	1872
gaa aag tgc ctc agc gta gcc tgc ctg gac aag aat gag ctc agt gac Glu Lys Cys Leu Ser Val Ala Cys Leu Asp Lys Asn Glu Leu Ser Asp 625 630 635 640	1920
cac ttg gat gct atg gac tcc aac ctg gat aac ctg cag acc atg ctg His Leu Asp Ala Met Asp Ser Asn Leu Asp Asn Leu Gln Thr Met Leu 645 650 655	1968
agc agc cac ggc ttc agc gtg gac acc agt gcc ctg ctg gac ctg ttc	2016

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Ser Ser His Gly Phe Ser Val Asp Thr Ser Ala Leu Leu Asp Leu Phe
      660                      665                      670

agc ccc tcg gtg acc gtg ccc gac atg agc ctg cct gac ctt gac agc 2064
Ser Pro Ser Val Thr Val Pro Asp Met Ser Leu Pro Asp Leu Asp Ser
      675                      680                      685

agc ctg gcc agt atc caa gag ctc ctg tct ccc cag gag ccc ccc agg 2112
Ser Leu Ala Ser Ile Gln Glu Leu Leu Ser Pro Gln Glu Pro Pro Arg
      690                      695                      700

cct ccc gag gca gag aac agc agc ccg gat tca ggg aag cag ctg gtg 2160
Pro Pro Glu Ala Glu Asn Ser Ser Pro Asp Ser Gly Lys Gln Leu Val
      705                      710                      715                      720

cac tac aca gcg cag ccg ctg ttc ctg ctg gac ccc ggc tcc gtg gac 2208
His Tyr Thr Ala Gln Pro Leu Phe Leu Leu Asp Pro Gly Ser Val Asp
      725                      730                      735

acc ggg agc aac gac ctg ccg gtg ctg ttt gag ctg gga gag ggc tcc 2256
Thr Gly Ser Asn Asp Leu Pro Val Leu Phe Glu Leu Gly Glu Gly Ser
      740                      745                      750

tac ttc tcc gaa ggg gac ggc ttc gcc gag gac ccc acc atc tcc ctg 2304
Tyr Phe Ser Glu Gly Asp Gly Phe Ala Glu Asp Pro Thr Ile Ser Leu
      755                      760                      765

ctg aca ggc tcg gag cct ccc aaa gcc aag gac ccc act gtc tcc 2349
Leu Thr Gly Ser Glu Pro Pro Lys Ala Lys Asp Pro Thr Val Ser
      770                      775                      780

tagaggcccc ggaggagctg ggccagccgc ccacccccac cccagtgca gggctgggtct 2409

tggggaggca gggcagcctc gcggtcttgg gcaactggtgg gtcggccgg 2458

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<210> 176

<211> 783

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-HSF1

<400> 176

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
  1                      5                      10                      15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
      20                      25                      30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
      35                      40                      45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
      50                      55                      60

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Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
 165 170 175
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190
 Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
 225 230 235 240
 Gly Leu Arg Ser Arg Ala Gln Ala Ser Asn Ser Ala Val Glu Met Asp
 245 250 255
 Leu Pro Val Gly Pro Gly Ala Ala Gly Pro Ser Asn Val Pro Ala Phe
 260 265 270
 Leu Thr Lys Leu Trp Thr Leu Val Ser Asp Pro Asp Thr Asp Ala Leu
 275 280 285
 Ile Cys Trp Ser Pro Ser Gly Asn Ser Phe His Val Phe Asp Gln Gly
 290 295 300
 Gln Phe Ala Lys Glu Val Leu Pro Lys Tyr Phe Lys His Asn Asn Met
 305 310 315 320
 Ala Ser Phe Val Arg Gln Leu Asn Met Tyr Gly Phe Arg Lys Val Val
 325 330 335
 His Ile Glu Gln Gly Gly Leu Val Lys Pro Glu Arg Asp Asp Thr Glu
 340 345 350
 Phe Gln His Pro Cys Phe Leu Arg Gly Gln Glu Gln Leu Leu Glu Asn
 355 360 365

Ile Lys Arg Lys Val Thr Ser Val Ser Thr Leu Lys Ser Glu Asp Ile
 370 375 380
 Lys Ile Arg Gln Asp Ser Val Thr Lys Leu Leu Thr Asp Val Gln Leu
 385 390 395 400
 Met Lys Gly Lys Gln Glu Cys Met Asp Ser Lys Leu Leu Ala Met Lys
 405 410 415
 His Glu Asn Glu Ala Leu Trp Arg Glu Val Ala Ser Leu Arg Gln Lys
 420 425 430
 His Ala Gln Gln Gln Lys Val Val Asn Lys Leu Ile Gln Phe Leu Ile
 435 440 445
 Ser Leu Val Gln Ser Asn Arg Ile Leu Gly Val Lys Arg Lys Ile Pro
 450 455 460
 Leu Met Leu Asn Asp Ser Gly Ser Ala His Ser Met Pro Lys Tyr Ser
 465 470 475 480
 Arg Gln Phe Ser Leu Glu His Val His Gly Ser Gly Pro Tyr Ser Ala
 485 490 495
 Pro Ser Pro Ala Tyr Ser Ser Ser Ser Leu Tyr Ala Pro Asp Ala Val
 500 505 510
 Ala Ser Ser Gly Pro Ile Ile Ser Asp Ile Thr Glu Leu Ala Pro Ala
 515 520 525
 Ser Pro Met Ala Ser Pro Gly Gly Ser Ile Asp Glu Arg Pro Leu Ser
 530 535 540
 Ser Ser Pro Leu Val Arg Val Lys Glu Glu Pro Pro Ser Pro Pro Gln
 545 550 555 560
 Ser Pro Arg Val Glu Glu Ala Ser Pro Gly Arg Pro Ser Ser Val Asp
 565 570 575
 Thr Leu Leu Ser Pro Thr Ala Leu Ile Asp Ser Ile Leu Arg Glu Ser
 580 585 590
 Glu Pro Ala Pro Ala Ser Val Thr Ala Leu Thr Asp Ala Arg Gly His
 595 600 605
 Thr Asp Thr Glu Gly Arg Pro Pro Ser Pro Pro Pro Thr Ser Thr Pro
 610 615 620
 Glu Lys Cys Leu Ser Val Ala Cys Leu Asp Lys Asn Glu Leu Ser Asp
 625 630 635 640
 His Leu Asp Ala Met Asp Ser Asn Leu Asp Asn Leu Gln Thr Met Leu
 645 650 655
 Ser Ser His Gly Phe Ser Val Asp Thr Ser Ala Leu Leu Asp Leu Phe
 660 665 670

Ser Pro Ser Val Thr Val Pro Asp Met Ser Leu Pro Asp Leu Asp Ser
675 680 685

Ser Leu Ala Ser Ile Gln Glu Leu Leu Ser Pro Gln Glu Pro Pro Arg
690 695 700

Pro Pro Glu Ala Glu Asn Ser Ser Pro Asp Ser Gly Lys Gln Leu Val
705 710 715 720

His Tyr Thr Ala Gln Pro Leu Phe Leu Leu Asp Pro Gly Ser Val Asp
725 730 735

Thr Gly Ser Asn Asp Leu Pro Val Leu Phe Glu Leu Gly Glu Gly Ser
740 745 750

Tyr Phe Ser Glu Gly Asp Gly Phe Ala Glu Asp Pro Thr Ile Ser Leu
755 760 765

Leu Thr Gly Ser Glu Pro Pro Lys Ala Lys Asp Pro Thr Val Ser
770 775 780

<210> 177

<211> 2416

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-NFKB

<220>

<221> CDS

<222> (1)..(2415)

<400> 177

atg	gtg	agc	aag	ggc	gag	gag	ctg	ttc	acc	ggg	gtg	gtg	ccc	atc	ctg	48
Met	Val	Ser	Lys	Gly	Glu	Glu	Leu	Phe	Thr	Gly	Val	Val	Pro	Ile	Leu	
1				5				10					15			
gtc	gag	ctg	gac	ggc	gac	gta	aac	ggc	cac	aag	ttc	agc	gtg	tcc	ggc	96
Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	
			20					25					30			
gag	ggc	gag	ggc	gat	gcc	acc	tac	ggc	aag	ctg	acc	ctg	aag	ttc	atc	144
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	
			35				40					45				
tgc	acc	acc	ggc	aag	ctg	ccc	gtg	ccc	tgg	ccc	acc	ctc	gtg	acc	acc	192
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	
	50					55				60						
ctg	acc	tac	ggc	gtg	cag	tgc	ttc	agc	cgc	tac	ccc	gac	cac	atg	aag	240
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	
65					70				75						80	
cag	cac	gac	ttc	ttc	aag	tcc	gcc	atg	ccc	gaa	ggc	tac	gtc	cag	gag	288
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	

85										90										95										
cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc gag	336																													
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu																														
100 105 110																														
gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc	384																													
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly																														
115 120 125																														
atc gac ttc aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac	432																													
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr																														
130 135 140																														
aac tac aac agc cac aac gtc tat atc atg gcc gac aag cag aag aac	480																													
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn																														
145 150 155 160																														
ggc atc aag gtg aac ttc aag atc cgc cac aac atc gag gac ggc agc	528																													
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser																														
165 170 175																														
gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac ggc	576																													
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly																														
180 185 190																														
ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg	624																													
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu																														
195 200 205																														
agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc	672																													
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe																														
210 215 220																														
gtg acc gcc gcc ggg atc act ctc ggc atg gac gag ctg tac aag tcc	720																													
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser																														
225 230 235 240																														
gga ctc aga tct cga gat ccg ccc ttc atg gac gaa ctg ttc ccc ctc	768																													
Gly Leu Arg Ser Arg Asp Pro Pro Phe Met Asp Glu Leu Phe Pro Leu																														
245 250 255																														
atc ttc ccg gca gag cca gcc cag gcc tct ggc ccc tat gtg gag atc	816																													
Ile Phe Pro Ala Glu Pro Ala Gln Ala Ser Gly Pro Tyr Val Glu Ile																														
260 265 270																														
att gag cag ccc aag cag cgg ggc atg cgc ttc cgc tac aag tgc gag	864																													
Ile Glu Gln Pro Lys Gln Arg Gly Met Arg Phe Arg Tyr Lys Cys Glu																														
275 280 285																														
ggg cgc tcc gcg ggc agc atc cca gcc gag agg agc aca gat acc acc	912																													
Gly Arg Ser Ala Gly Ser Ile Pro Gly Glu Arg Ser Thr Asp Thr Thr																														
290 295 300																														
aag acc cac ccc acc atc aag atc aat ggc tac aca gga cca ggg aca	960																													
Lys Thr His Pro Thr Ile Lys Ile Asn Gly Tyr Thr Gly Pro Gly Thr																														
305 310 315 320																														

gtg cgc atc tcc ctg gtc acc aag gac cct cct cac cgg cct cac ccc	1008
Val Arg Ile Ser Leu Val Thr Lys Asp Pro Pro His Arg Pro His Pro	
325 330 335	
cac gag ctt gta gga aag gac tgc cgg gat ggc ttc tat gag gct gag	1056
His Glu Leu Val Gly Lys Asp Cys Arg Asp Gly Phe Tyr Glu Ala Glu	
340 345 350	
ctc tgc ccg gac cgc tgc atc cac agt ttc cag aac ctg gga atc cag	1104
Leu Cys Pro Asp Arg Cys Ile His Ser Phe Gln Asn Leu Gly Ile Gln	
355 360 365	
tgt gtg aag aag cgg gac ctg gag cag gct atc agt cag cgc atc cag	1152
Cys Val Lys Lys Arg Asp Leu Glu Gln Ala Ile Ser Gln Arg Ile Gln	
370 375 380	
acc aac aac aac ccc ttc caa gtt cct ata gaa gag cag cgt ggg gac	1200
Thr Asn Asn Asn Pro Phe Gln Val Pro Ile Glu Glu Gln Arg Gly Asp	
385 390 395 400	
tac gac ctg aat gct gtg cgg ctc tgc ttc cag gtg aca gtg cgg gac	1248
Tyr Asp Leu Asn Ala Val Arg Leu Cys Phe Gln Val Thr Val Arg Asp	
405 410 415	
cca tca ggc agg ccc ctc cgc ctg ccg cct gtc ctt tct cat ccc atc	1296
Pro Ser Gly Arg Pro Leu Arg Leu Pro Pro Val Leu Ser His Pro Ile	
420 425 430	
ttt gac aat cgt gcc ccc aac act gcc gag ctc aag atc tgc cga gtg	1344
Phe Asp Asn Arg Ala Pro Asn Thr Ala Glu Leu Lys Ile Cys Arg Val	
435 440 445	
aac cga aac tct ggc agc tgc ctc ggt ggg gat gag atc ttc cta ctg	1392
Asn Arg Asn Ser Gly Ser Cys Leu Gly Gly Asp Glu Ile Phe Leu Leu	
450 455 460	
tgt gac aag gtg cag aaa gag gac att gag gtg tat ttc acg gga cca	1440
Cys Asp Lys Val Gln Lys Glu Asp Ile Glu Val Tyr Phe Thr Gly Pro	
465 470 475 480	
ggc tgg gag gcc cga ggc tcc ttt tcg caa gct gat gtg cac cga caa	1488
Gly Trp Glu Ala Arg Gly Ser Phe Ser Gln Ala Asp Val His Arg Gln	
485 490 495	
gtg gcc att gtg ttc cgg acc cct ccc tac gca gac ccc agc ctg cag	1536
Val Ala Ile Val Phe Arg Thr Pro Pro Tyr Ala Asp Pro Ser Leu Gln	
500 505 510	
gct cct gtg cgt gtc tcc atg cag ctg cgg cgg cct tcc gac cgg gag	1584
Ala Pro Val Arg Val Ser Met Gln Leu Arg Arg Pro Ser Asp Arg Glu	
515 520 525	
ctc agt gag ccc atg gaa ttc cag tac ctg cca gat aca gac gat cgt	1632
Leu Ser Glu Pro Met Glu Phe Gln Tyr Leu Pro Asp Thr Asp Asp Arg	
530 535 540	

cac cgg att gag gag aaa cgt aaa agg aca tat gag acc ttc aag agc	1680
His Arg Ile Glu Glu Lys Arg Lys Arg Thr Tyr Glu Thr Phe Lys Ser	
545 550 555 560	
atc atg aag aag agt cct ttc agc gga ccc acc gac ccc cgg cct cca	1728
Ile Met Lys Lys Ser Pro Phe Ser Gly Pro Thr Asp Pro Arg Pro Pro	
565 570 575	
cct cga cgc att gct gtg cct tcc cgc agc tca gct tct gtc ccc aag	1776
Pro Arg Arg Ile Ala Val Pro Ser Arg Ser Ser Ala Ser Val Pro Lys	
580 585 590	
cca gca ccc cag ccc tat ccc ttt acg tca tcc ctg agc acc atc aac	1824
Pro Ala Pro Gln Pro Tyr Pro Phe Thr Ser Ser Leu Ser Thr Ile Asn	
595 600 605	
tat gat gag ttt ccc acc atg gtg ttt cct tct ggg cag atc agc cag	1872
Tyr Asp Glu Phe Pro Thr Met Val Phe Pro Ser Gly Gln Ile Ser Gln	
610 615 620	
gcc tcg gcc ttg gcc ccg gcc cct ccc caa gtc ctg ccc cag gct cca	1920
Ala Ser Ala Leu Ala Pro Ala Pro Pro Gln Val Leu Pro Gln Ala Pro	
625 630 635 640	
gcc cct gcc cct gct cca gcc atg gta tca gct ctg gcc cag gcc cca	1968
Ala Pro Ala Pro Ala Pro Ala Met Val Ser Ala Leu Ala Gln Ala Pro	
645 650 655	
gcc cct gtc cca gtc cta gcc cca ggc cct cct cag gct gtg gcc cca	2016
Ala Pro Val Pro Val Leu Ala Pro Gly Pro Pro Gln Ala Val Ala Pro	
660 665 670	
cct gcc ccc aag ccc acc cag gct ggg gaa gga acg ctg tca gag gcc	2064
Pro Ala Pro Lys Pro Thr Gln Ala Gly Glu Gly Thr Leu Ser Glu Ala	
675 680 685	
ctg ctg cag ctg cag ttt gat gat gaa gac ctg ggg gcc ttg ctt ggc	2112
Leu Leu Gln Leu Gln Phe Asp Asp Glu Asp Leu Gly Ala Leu Leu Gly	
690 695 700	
aac agc aca gac cca gct gtg ttc aca gac ctg gca tcc gtc gac aac	2160
Asn Ser Thr Asp Pro Ala Val Phe Thr Asp Leu Ala Ser Val Asp Asn	
705 710 715 720	
tcc gag ttt cag cag ctg ctg aac cag ggc ata cct gtg gcc ccc cac	2208
Ser Glu Phe Gln Gln Leu Leu Asn Gln Gly Ile Pro Val Ala Pro His	
725 730 735	
aca act gag ccc atg ctg atg gag tac cct gag gct ata act cgc cta	2256
Thr Thr Glu Pro Met Leu Met Glu Tyr Pro Glu Ala Ile Thr Arg Leu	
740 745 750	
gtg aca gcc cag agg ccc ccc gac cca gct cct gct cca ctg ggg gcc	2304
Val Thr Ala Gln Arg Pro Pro Asp Pro Ala Pro Ala Pro Leu Gly Ala	
755 760 765	
ccg ggg ctc ccc aat ggc ctc ctt tca gga gat gaa gac ttc tcc tcc	2352

Pro Gly Leu Pro Asn Gly Leu Leu Ser Gly Asp Glu Asp Phe Ser Ser
 770 775 780
 att gcg gac atg gac ttc tca gcc ctg ctg agt cag atc agc tcc aag 2400
 Ile Ala Asp Met Asp Phe Ser Ala Leu Leu Ser Gln Ile Ser Ser Lys
 785 790 795 800
 ggc gaa ttc gaa gct t
 Gly Glu Phe Glu Ala 2416
 805

<210> 178
 <211> 805
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: GFP-NFKB

<400> 178
 Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1 5 10 15
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
 20 25 30
 Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
 35 40 45
 Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
 50 55 60
 Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
 65 70 75 80
 Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
 85 90 95
 Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
 100 105 110
 Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
 115 120 125
 Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
 130 135 140
 Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
 145 150 155 160
 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
 165 170 175
 Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
 180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
 195 200 205
 Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
 210 215 220
 Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Ser
 225 230 235 240
 Gly Leu Arg Ser Arg Asp Pro Pro Phe Met Asp Glu Leu Phe Pro Leu
 245 250 255
 Ile Phe Pro Ala Glu Pro Ala Gln Ala Ser Gly Pro Tyr Val Glu Ile
 260 265 270
 Ile Glu Gln Pro Lys Gln Arg Gly Met Arg Phe Arg Tyr Lys Cys Glu
 275 280 285
 Gly Arg Ser Ala Gly Ser Ile Pro Gly Glu Arg Ser Thr Asp Thr Thr
 290 295 300
 Lys Thr His Pro Thr Ile Lys Ile Asn Gly Tyr Thr Gly Pro Gly Thr
 305 310 315 320
 Val Arg Ile Ser Leu Val Thr Lys Asp Pro Pro His Arg Pro His Pro
 325 330 335
 His Glu Leu Val Gly Lys Asp Cys Arg Asp Gly Phe Tyr Glu Ala Glu
 340 345 350
 Leu Cys Pro Asp Arg Cys Ile His Ser Phe Gln Asn Leu Gly Ile Gln
 355 360 365
 Cys Val Lys Lys Arg Asp Leu Glu Gln Ala Ile Ser Gln Arg Ile Gln
 370 375 380
 Thr Asn Asn Asn Pro Phe Gln Val Pro Ile Glu Glu Gln Arg Gly Asp
 385 390 395 400
 Tyr Asp Leu Asn Ala Val Arg Leu Cys Phe Gln Val Thr Val Arg Asp
 405 410 415
 Pro Ser Gly Arg Pro Leu Arg Leu Pro Pro Val Leu Ser His Pro Ile
 420 425 430
 Phe Asp Asn Arg Ala Pro Asn Thr Ala Glu Leu Lys Ile Cys Arg Val
 435 440 445
 Asn Arg Asn Ser Gly Ser Cys Leu Gly Gly Asp Glu Ile Phe Leu Leu
 450 455 460
 Cys Asp Lys Val Gln Lys Glu Asp Ile Glu Val Tyr Phe Thr Gly Pro
 465 470 475 480
 Gly Trp Glu Ala Arg Gly Ser Phe Ser Gln Ala Asp Val His Arg Gln
 485 490 495

Val Ala Ile Val Phe Arg Thr Pro Pro Tyr Ala Asp Pro Ser Leu Gln
 500 505 510
 Ala Pro Val Arg Val Ser Met Gln Leu Arg Arg Pro Ser Asp Arg Glu
 515 520 525
 Leu Ser Glu Pro Met Glu Phe Gln Tyr Leu Pro Asp Thr Asp Asp Arg
 530 535 540
 His Arg Ile Glu Glu Lys Arg Lys Arg Thr Tyr Glu Thr Phe Lys Ser
 545 550 555 560
 Ile Met Lys Lys Ser Pro Phe Ser Gly Pro Thr Asp Pro Arg Pro Pro
 565 570 575
 Pro Arg Arg Ile Ala Val Pro Ser Arg Ser Ser Ala Ser Val Pro Lys
 580 585 590
 Pro Ala Pro Gln Pro Tyr Pro Phe Thr Ser Ser Leu Ser Thr Ile Asn
 595 600 605
 Tyr Asp Glu Phe Pro Thr Met Val Phe Pro Ser Gly Gln Ile Ser Gln
 610 615 620
 Ala Ser Ala Leu Ala Pro Ala Pro Pro Gln Val Leu Pro Gln Ala Pro
 625 630 635 640
 Ala Pro Ala Pro Ala Pro Ala Met Val Ser Ala Leu Ala Gln Ala Pro
 645 650 655
 Ala Pro Val Pro Val Leu Ala Pro Gly Pro Pro Gln Ala Val Ala Pro
 660 665 670
 Pro Ala Pro Lys Pro Thr Gln Ala Gly Glu Gly Thr Leu Ser Glu Ala
 675 680 685
 Leu Leu Gln Leu Gln Phe Asp Asp Glu Asp Leu Gly Ala Leu Leu Gly
 690 695 700
 Asn Ser Thr Asp Pro Ala Val Phe Thr Asp Leu Ala Ser Val Asp Asn
 705 710 715 720
 Ser Glu Phe Gln Gln Leu Leu Asn Gln Gly Ile Pro Val Ala Pro His
 725 730 735
 Thr Thr Glu Pro Met Leu Met Glu Tyr Pro Glu Ala Ile Thr Arg Leu
 740 745 750
 Val Thr Ala Gln Arg Pro Pro Asp Pro Ala Pro Ala Pro Leu Gly Ala
 755 760 765
 Pro Gly Leu Pro Asn Gly Leu Leu Ser Gly Asp Glu Asp Phe Ser Ser
 770 775 780
 Ile Ala Asp Met Asp Phe Ser Ala Leu Leu Ser Gln Ile Ser Ser Lys
 785 790 795 800

Gly Glu Phe Glu Ala
805

<210> 179

<211> 1677

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-IKB

<220>

<221> CDS

<222> (1)..(1674)

<400> 179

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Met Phe Gln Ala Ala Glu Arg Pro Gln Glu Trp Ala Met Glu Gly Pro	
1 5 10 15	
cgc gac ggg ctg aag aag gag cgg cta ctg gac gac cgc cac gac agc	96
Arg Asp Gly Leu Lys Lys Glu Arg Leu Leu Asp Asp Arg His Asp Ser	
20 25 30	
ggc ctg gac tcc atg aaa gac gag gag tac gag cag atg gtc aag gag	144
Gly Leu Asp Ser Met Lys Asp Glu Glu Tyr Glu Gln Met Val Lys Glu	
35 40 45	
ctg cag gag atc cgc ctc gag ccg cag gag gtg ccg cgc ggc tcg gag	192
Leu Gln Glu Ile Arg Leu Glu Pro Gln Glu Val Pro Arg Gly Ser Glu	
50 55 60	
ccc tgg aag cag cag ctc acc gag gac ggg gac tcg ttc ctg cac ttg	240
Pro Trp Lys Gln Gln Leu Thr Glu Asp Gly Asp Ser Phe Leu His Leu	
65 70 75 80	
gcc atc atc cat gaa gaa aag gca ctg acc atg gaa gtg atc cgc cag	288
Ala Ile Ile His Glu Glu Lys Ala Leu Thr Met Glu Val Ile Arg Gln	
85 90 95	
gtg aag gga gac ctg gcc ttc ctc aac ctc cag aac aac ctg cag cag	336
Val Lys Gly Asp Leu Ala Phe Leu Asn Leu Gln Asn Asn Leu Gln Gln	
100 105 110	
act cca ctc cac ttg gct gtg atc acc aac cag cca gaa att gct gag	384
Thr Pro Leu His Leu Ala Val Ile Thr Asn Gln Pro Glu Ile Ala Glu	
115 120 125	
gca ctt ctg gga gct ggc tgt gat cct gag ctc cga gac ttt cga gga	432
Ala Leu Leu Gly Ala Gly Cys Asp Pro Glu Leu Arg Asp Phe Arg Gly	
130 135 140	
aat acc ccc cta cac ctt gcc tgt gag cag ggc tgc ctg gcc agc gtg	480
Asn Thr Pro Leu His Leu Ala Cys Glu Gln Gly Cys Leu Ala Ser Val	
145 150 155 160	

gga gtc ctg act cag tcc tgc acc acc ccg cac ctc cac tcc atc ttg	528
Gly Val Leu Thr Gln Ser Cys Thr Thr Pro His Leu His Ser Ile Leu	
165 170 175	
aag gct acc aac tac aat ggc cac acg tgt cta cac tta gcc tct atc	576
Lys Ala Thr Asn Tyr Asn Gly His Thr Cys Leu His Leu Ala Ser Ile	
180 185 190	
cat ggc tac ctg ggc atc gtg gag ctt ttg gtg tcc ttg ggt gct gat	624
His Gly Tyr Leu Gly Ile Val Glu Leu Leu Val Ser Leu Gly Ala Asp	
195 200 205	
gtc aat gct cag gag ccc tgt aat ggc cgg act gcc ctt cac ctc gca	672
Val Asn Ala Gln Glu Pro Cys Asn Gly Arg Thr Ala Leu His Leu Ala	
210 215 220	
gtg gac ctg caa aat cct gac ctg gtg tca ctc ctg ttg aag tgt ggg	720
Val Asp Leu Gln Asn Pro Asp Leu Val Ser Leu Leu Leu Lys Cys Gly	
225 230 235 240	
gct gat gtc aac aga gtt acc tac cag ggc tat tct ccc tac cag ctc	768
Ala Asp Val Asn Arg Val Thr Tyr Gln Gly Tyr Ser Pro Tyr Gln Leu	
245 250 255	
acc tgg ggc cgc cca agc acc cgg ata cag cag cag ctg ggc cag ctg	816
Thr Trp Gly Arg Pro Ser Thr Arg Ile Gln Gln Gln Leu Gly Gln Leu	
260 265 270	
aca cta gaa aac ctt cag atg ctg cca gag agt gag gat gag gag agc	864
Thr Leu Glu Asn Leu Gln Met Leu Pro Glu Ser Glu Asp Glu Glu Ser	
275 280 285	
tat gac aca gag tca gag ttc acg gag ttc aca gag gac gag ctg ccc	912
Tyr Asp Thr Glu Ser Glu Phe Thr Glu Phe Thr Glu Asp Glu Leu Pro	
290 295 300	
tat gat gac tgt gtg ttt gga ggc cag cgt ctg acg tta acc ggt atg	960
Tyr Asp Asp Cys Val Phe Gly Gly Gln Arg Leu Thr Leu Thr Gly Met	
305 310 315 320	
gct agc aaa gga gaa gaa ctc ttc act gga gtt gtc cca att ctt gtt	1008
Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val	
325 330 335	
gaa tta gat ggt gat gtt aac ggc cac aag ttc tct gtc agt gga gag	1056
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu	
340 345 350	
ggt gaa ggt gat gca aca tac gga aaa ctt acc ctg aag ttc atc tgc	1104
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys	
355 360 365	
act act ggc aaa ctg cct gtt cca tgg cca aca cta gtc act act ctg	1152
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Leu	
370 375 380	
tgc tat ggt gtt caa tgc ttt tca aga tac ccg gat cat atg aaa cgg	1200

Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	Arg		
385					390					395					400		
cat	gac	ttt	ttc	aag	agt	gcc	atg	ccc	gaa	ggg	tat	gta	cag	gaa	agg	1248	
His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	Arg		
				405					410					415			
acc	atc	ttc	ttc	aaa	gat	gac	ggc	aac	tac	aag	aca	cgt	gct	gaa	gtc	1296	
Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	Val		
				420				425					430				
aag	ttt	gaa	ggg	gat	acc	ctt	gtt	aat	aga	atc	gag	tta	aaa	ggg	att	1344	
Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile		
		435					440					445					
gac	ttc	aag	gaa	gat	ggc	aac	att	ctg	gga	cac	aaa	ttg	gaa	tac	aac	1392	
Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn		
		450				455					460						
tat	aac	tca	cac	aat	gta	tac	atc	atg	gca	gac	aaa	caa	aag	aat	gga	1440	
Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	Gly		
					470				475						480		
atc	aaa	gtg	aac	ttc	aag	acc	cgc	cac	aac	att	gaa	gat	gga	agc	gtt	1488	
Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	Val		
				485					490					495			
caa	cta	gca	gac	cat	tat	caa	caa	aat	act	cca	att	ggc	gat	ggc	cct	1536	
Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	Pro		
				500				505					510				
gtc	ctt	tta	cca	gac	aac	cat	tac	ctg	tcc	aca	caa	tct	gcc	ctt	tcg	1584	
Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	Ser		
				515				520					525				
aaa	gat	ccc	aac	gaa	aag	aga	gac	cac	atg	gtc	ctt	ctt	gag	ttt	gta	1632	
Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	Val		
		530				535					540						
aca	gct	gct	ggg	att	aca	cat	ggc	atg	gat	gaa	ctg	tac	aac	tag		1677	
Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Asn				
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<210> 180

<211> 558

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: GFP-IKB

<400> 180

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Arg	Asp	Gly	Leu	Lys	Lys	Glu	Arg	Leu	Leu	Asp	Asp	Arg	His	Asp	Ser
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

20 25 30
 Gly Leu Asp Ser Met Lys Asp Glu Glu Tyr Glu Gln Met Val Lys Glu
 35 40 45
 Leu Gln Glu Ile Arg Leu Glu Pro Gln Glu Val Pro Arg Gly Ser Glu
 50 55 60
 Pro Trp Lys Gln Gln Leu Thr Glu Asp Gly Asp Ser Phe Leu His Leu
 65 70 75 80
 Ala Ile Ile His Glu Glu Lys Ala Leu Thr Met Glu Val Ile Arg Gln
 85 90 95
 Val Lys Gly Asp Leu Ala Phe Leu Asn Leu Gln Asn Asn Leu Gln Gln
 100 105 110
 Thr Pro Leu His Leu Ala Val Ile Thr Asn Gln Pro Glu Ile Ala Glu
 115 120 125
 Ala Leu Leu Gly Ala Gly Cys Asp Pro Glu Leu Arg Asp Phe Arg Gly
 130 135 140
 Asn Thr Pro Leu His Leu Ala Cys Glu Gln Gly Cys Leu Ala Ser Val
 145 150 155 160
 Gly Val Leu Thr Gln Ser Cys Thr Thr Pro His Leu His Ser Ile Leu
 165 170 175
 Lys Ala Thr Asn Tyr Asn Gly His Thr Cys Leu His Leu Ala Ser Ile
 180 185 190
 His Gly Tyr Leu Gly Ile Val Glu Leu Leu Val Ser Leu Gly Ala Asp
 195 200 205
 Val Asn Ala Gln Glu Pro Cys Asn Gly Arg Thr Ala Leu His Leu Ala
 210 215 220
 Val Asp Leu Gln Asn Pro Asp Leu Val Ser Leu Leu Leu Lys Cys Gly
 225 230 235 240
 Ala Asp Val Asn Arg Val Thr Tyr Gln Gly Tyr Ser Pro Tyr Gln Leu
 245 250 255
 Thr Trp Gly Arg Pro Ser Thr Arg Ile Gln Gln Gln Leu Gly Gln Leu
 260 265 270
 Thr Leu Glu Asn Leu Gln Met Leu Pro Glu Ser Glu Asp Glu Glu Ser
 275 280 285
 Tyr Asp Thr Glu Ser Glu Phe Thr Glu Phe Thr Glu Asp Glu Leu Pro
 290 295 300
 Tyr Asp Asp Cys Val Phe Gly Gly Gln Arg Leu Thr Leu Thr Gly Met
 305 310 315 320
 Ala Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val

325										330					335				
Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	Glu				
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		355					360					365							
Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	Leu				
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Cys	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	Arg				
385					390				395					400					
His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	Arg				
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Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	Val				
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Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile				
	435					440					445								
Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn				
	450				455					460									
Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	Gly				
465				470					475					480					
Ile	Lys	Val	Asn	Phe	Lys	Thr	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	Val				
			485				490						495						
Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	Pro				
			500				505					510							
Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	Ser				
	515					520					525								
Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	Val				
	530				535				540										
Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Asn						
545				550					555										

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/04794

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N15/14 G01N33/50 C12M1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 45704 A (TULLIN SOEREN ;KASPER ALMHOLT (DK); NOVONORDISK AS (DK); SCUDDER K) 15 October 1998 (1998-10-15) abstract; figures 1,19	1,3,20
A	WO 97 38127 A (GEN HOSPITAL CORP ;GENETICS INST (US)) 16 October 1997 (1997-10-16) page 1, line 13 page 4, line 14 -page 5, line 17 page 12, line 9-15 --- -/--	1,3,20

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

27 October 2000

Date of mailing of the international search report

13. 11. 00

Name and mailing address of the ISA

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Authorized officer

Zinngrebe, U

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/04794

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>GIULIANO K A ET AL: "Fluorescent-protein biosensors: new tools for drug discovery" TRENDS IN BIOTECHNOLOGY, GB, ELSEVIER PUBLICATIONS, CAMBRIDGE, vol. 16, no. 3, 1 March 1998 (1998-03-01), pages 135-140, XP004108592 ISSN: 0167-7799 page 138, column 2, last paragraph page 139, column 2, paragraph 2 -----</p>	1,3,20
A	<p>WO 98 38490 A (BIODX INC ;DUNLAY R TERRY (US); GOUGH ALBERT H (US); GIULIANO KENN) 3 September 1998 (1998-09-03) abstract page 7, line 27 -page 8, line 10 -----</p>	1,3,20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/04794

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 17
because they relate to subject matter not required to be searched by this Authority, namely:
Rule 39.1(vi) PCT - Program for computers
2. ☒ Claims Nos.: 18,19
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 18,19

there is lack of clarity as it is unclear if the instructions detailing the method are to be used to define the kit (not allowable as a method may not be used to define a product) or alternatively were to be regarded as a sheet of paper. F

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 00/04794

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9845704 A	15-10-1998	AU 6820998 A EP 0986753 A	30-10-1998 22-03-2000
WO 9738127 A	16-10-1997	EP 0904402 A JP 2000508174 T	31-03-1999 04-07-2000
WO 9838490 A	03-09-1998	US 5989835 A US 6103479 A AU 6667898 A EP 0983498 A JP 2000509827 T AU 3297197 A EP 0912892 A	23-11-1999 15-08-2000 18-09-1998 08-03-2000 02-08-2000 05-01-1998 06-05-1999

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